

# IN SITU HYBRIDISATION FOR THE DETECTION OF VIRAL NUCLEIC ACIDS

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***IN SITU* HYBRIDISATION FOR THE DETECTION  
OF VIRAL NUCLEIC ACIDS.**

Jane Anthea Hoyle.

Submitted in accordance with the requirements  
for the degree of Doctor of Philosophy.

The University of St. Andrews.  
Department of Biochemistry and Microbiology.

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**ABSTRACT.**

The technique of *in situ* hybridisation was optimised for the detection of viral RNA using radioactively-labelled single-stranded DNA and RNA probes, and applied to three areas of interest.

Optimum hybridisation conditions were determined *in vitro* using cells infected with the single-stranded negative sense RNA paramyxoviruses. Transcription of RNA probes was the most rapid and efficient method of probe labelling, since electrophoretic purification was not required and large amounts of RNA were produced. However, their use for *in situ* hybridisation was problematic due to RNase contamination and low sensitivity. In contrast, DNA probes produced from M13 clones and oligonucleotide probes gave consistent hybridisation results and were preferred in subsequent studies for their ease of use, stability and sensitivity.

The effect of virus-host interactions on the clearance of the paramyxovirus, SV5, in a mouse model was investigated by detection of viral RNA and protein in lung sections. Immunisation with purified SV5 proteins prior to infection provided protection against infection, indicated by a reduction in the level of viral RNA and protein, due to enhanced clearance of virus by primed T cells. X-irradiation of the host prior to infection resulted in prolonged or persistent infection in which RNA was detected up to 19 days post-infection.

The potential of *in situ* hybridisation for detection of aetiological agents was demonstrated by investigation of the presence of measles virus in two chronic human diseases. Thus, measles virus RNA was detected in brain sections from a patient with subacute sclerosing panencephalitis and in the osteoclasts of bone sections from a patient with Paget's disease of bone.

*In situ* hybridisation was used to analyse expression of the two immediate-early genes of herpesvirus saimiri, the 52K gene and the hinG gene. Differential expression was detected by hybridisation to mRNA using oligonucleotide probes, in productively-infected cells. The 52K gene was expressed asynchronously throughout the population in agreement with immunocytochemical detection of the 52K protein. In contrast, the hinG gene was expressed synchronously, with all cells showing similar levels of hybridisation, indicating a specific control mechanism for expression of the 52K gene, which differs from that of the hinG gene in requiring or being inhibited by additional factors. This may have relevance to the mechanism of establishment of latency in this virus.

## OUTLINE OF THESIS STRUCTURE.

The overall aim of the thesis was to optimise the technique of *in situ* hybridisation for the detection of viral RNA and apply it to areas of research interest within our laboratory.

In Section One, the technique has been analysed in detail. In Chapter One, the principles involved in *in situ* hybridisation are discussed. Chapters Two and Three describe the choice of probes for hybridisation and experiments undertaken to optimise hybridisation conditions using these probes.

Section Two describes the application of *in situ* hybridisation to three areas of interest, using optimal conditions determined in Section One. In Chapter Four, *in situ* hybridisation has been applied to studies of the paramyxovirus, simian virus 5, using a mouse model system to analyse the effects of experimental perturbation of the host's immune system on viral replication. Chapter Five illustrates the use of *in situ* hybridisation for the detection of putative aetiological agents in chronic diseases, by examining the presence of measles virus RNA in two human diseases, subacute sclerosing panencephalitis and Paget's disease of bone. Finally, in Chapter Six, *in situ* hybridisation has been used to investigate differential regulation of gene expression in a herpesvirus at the single cell level *in vitro*.

In the final chapter, developments in the technique of *in situ* hybridisation and future applications are discussed.

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**DEDICATION.**

To my husband, Richard, for his constant love  
and support, and my parents for providing  
an education to make this possible.

## ABBREVIATIONS.

A	absorbance
Amp <sup>R</sup>	ampicillin resistance gene
ATP	adenosine triphosphate
bp	base pair
BPB	bromophenol blue
c.p.e.	cytopathic effect
Ci	Curie
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dH <sub>2</sub> O	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide
dpm	disintegrations per minute
dTTP	deoxythymidine triphosphate
G + C	guanosine + cytosine
h	hour
HEPES	N-2-hydroxyethylpiperidine-N'-2-ethanesulphonic acid
Ig	immunoglobulin
IPTG	isopropyl-1-thio-β-D-galactoside
kb	kilobase
kD	kilodalton
keV	kiloelectronvolt
mcs	multiple cloning site
min	minute
m.o.i.	multiplicity of infection
Mrads	megarads
mRNA	messenger RNA
NP40	Nonidet P-40
O.D.	optical density
OH	hydroxyl
ORF	open reading frame
P	phosphate

PBS'A'	phosphate-buffered saline
p.f.u.	plaque-forming units
p.i.	post-infection
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
rpm	revolutions per minute
Sp.act.	specific activity
Tris-HCl	tris-hydroxymethyl-aminomethane, pH adjusted with HCl
U	unit
UV	ultra-violet
V	volts
v/v	volume/volume
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside

## SECTION ONE.

In this section, the methodology of *in situ* hybridisation is described in detail. An introduction to the technique is given in Chapter One, followed in Chapter Two by a description of experiments undertaken to produce radioactively-labelled single-stranded DNA and RNA probes. In Chapter Three, the optimal hybridisation conditions for the detection of single-stranded viral RNA *in situ* have been characterised for each probe type.

**CHAPTER ONE.**

**Introduction to the technique of  
*in situ* hybridisation.**

### 1.1. INTRODUCTION TO *IN SITU* HYBRIDISATION.

The technique of *in situ* hybridisation has developed by extension of mixed phase or filter hybridisations, such as Southern and Northern blotting and involves hybridisation of a labelled nucleic acid probe to DNA or RNA within individual cells.

The technique has two main advantages over conventional hybridisation methods. Firstly, resolution of the hybridisation signal to individual cells allows regional localisation and identification of the cell types involved, which is of particular importance in highly heterogeneous tissues, such as the brain. Improvements in resolution have allowed subcellular localisation to be determined (nuclear or cytoplasmic), genes to be mapped to specific sites on individual chromosomes (Gerhard et al,1981; Harper et al,1981; Neel et al,1982; Trent et al,1982; Bhatt et al,1988) and ultrastructural localisation of hybridisation by electron microscopy (Hutchinson et al,1982; Binder et al,1986; Singer et al,1986; 1989; Webster et al,1987). Secondly, hybridisation *in situ* provides increased sensitivity in situations where the gene of interest is present in only a small proportion of the cells, since there is no extraction of nucleic acids and hence dilution of the gene of interest in the other cellular nucleic acids is avoided.

*In situ* hybridisation was first demonstrated by Gall and Pardue in 1969, when they detected the amplified rRNA genes in the "cap" region of the oocyte of *Xenopus laevis* (Gall & Pardue,1969; Gall & Pardue,1971). The technique has since been refined and developed by numerous workers for detection of both DNA and RNA, for a variety of applications (Brahic & Haase,1978; Moar & Klein,1978; Gee & Roberts,1983; Haase et al,1984a; Haase et al, 1985a; Lawrence & Singer, 1985; Berger,1986; Haase,1986). Advances in molecular biology have resulted in the production of increasingly more specific and sensitive probes allowing quantitative assays to be developed, with detectable target nucleic acid concentrations in the range of only a few copies per cell(Brahic & Haase,1978;Haase et al,1985b;Haase,1987).

The technique obviously has vast potential and is becoming widely used in



conjunction with conventional methods of DNA and RNA analysis in areas of research ranging from developmental biology and neurology to pathology, virology, and the search for new aetiological agents in disease.

*In situ* hybridisation has found particular application in the field of virology. Since most viral infections are highly focal with only very low numbers of cells being infected, the increase in sensitivity provided by *in situ* hybridisation over other hybridisation techniques allows detection of virus in such situations and has the additional advantage of allowing correlation of the presence of virus with pathological changes in the infected tissue.

Two examples in the field of virology that have greatly benefitted from advances in *in situ* hybridisation involve restricted gene expression. The first example is in the studies of the transcriptional state of viruses during latency, in particular the herpesviruses. *In situ* hybridisation has allowed both *in vitro* and *in vivo* studies to identify the specific cell types in which the latent infection is established, and identification of the transcripts produced. *In situ* hybridisation was particularly applicable to these studies, where only a small proportion of cells harbour the virus, and where viral proteins have not been detected under conditions of latency. Thus herpes simplex virus DNA was detected in neurons within sensory ganglia following explantation and cultivation (Stevens & Cook, 1971; Knotts et al, 1973), and the presence of virus in these cells was later confirmed by direct detection of RNA transcripts (Galloway et al, 1982; Tenser et al, 1982; Stroop et al, 1984; Rock et al, 1987; Stevens et al, 1987; 1988). By using probes specific to certain areas of the genome, it was shown that transcription was specifically restricted to an area of the genome in the long terminal repeat region, complementary to the IE ICPO gene, which was named the latency-associated transcript (LAT) (Croen et al, 1987; Rock et al, 1987; Gorden et al, 1988; Stevens et al, 1988). Further studies of this kind should help to elucidate mechanisms of latency in other herpesviruses, such as cytomegalovirus and varicella-zoster virus. In addition, *in situ* hybridisation can be

used to detect carriers of other infections, such as human immunodeficiency virus (HIV) (Pezzella et al,1989).

The second area of research where *in situ* hybridisation has found particular application is in the study of chronic diseases of viral aetiology. Since the realisation that many viruses can persist in tissue for long periods of time in the absence of detectable levels of viral proteins or clinical disease, interest has been revived in the potential pathogenic role of viruses in a number of chronic diseases. *In situ* hybridisation provides the ideal technique for detection of virus in such situations where gene expression is often severely restricted and the disease is highly focal. In addition to studies of the pathogenesis of diseases such as sub-acute sclerosing panencephalitis (SSPE) and progressive multifocal leucoencephalopathy (PML), where a viral aetiology has been established, *in situ* hybridisation has been used to search for putative aetiological agents in a number of other diseases of unknown cause, such as multiple sclerosis (Cosby et al,1989; Haase et al,1981b; 1985b). The ability of *in situ* hybridisation to reveal the presence of low levels of viral nucleic acids, whether transcriptionally active or not, should shed light on the possible pathogenic role played by such viruses.

*In situ* hybridisation has also been used to study the pathogenesis and spread of viral infections *in vivo* in experimental animal model systems, often suggesting possible mechanisms of virally-induced pathogenesis in human diseases (Brahic & Stroop,1981; Perlman et al,1988; Zurbriggen & Fujinami,1988; Lagarias & Radke,1989).

*In situ* hybridisation is thus a powerful molecular tool and is likely to find many applications in the field of virology. With further developments in sensitivity and the use of non-radioactive detection methods it should become a routine method for both research and detection of viruses in disease.

## 1.2. THEORETICAL PRINCIPLES OF NUCLEIC ACID HYBRIDISATION.

A general understanding of the factors affecting hybrid formation is useful for optimisation of hybridisation conditions. The factors affecting hybrid stability and the kinetics of hybridisation have been well characterised for nucleic acids in solution and for mixed phase or filter hybridisations. These are generally applicable to *in situ* hybridisation which is an example of mixed phase hybridisation where one nucleic acid is fixed in position and the other is in solution. Theoretical values can thus be used as a guideline when first selecting appropriate conditions for *in situ* hybridisation, although a more accurate experimental determination is also advisable. The major difference between *in situ* hybridisation and filter hybridisation is in the kinetics of the reaction which are affected by the requirement for the probe to penetrate the cell before hybridisation can occur.

### 1.2.1. Factors affecting hybrid stability.

The melting temperature ( $T_m$ ) of a hybrid is defined as the temperature at which half of the hybrids are dissociated under a given set of conditions, and is used as a measure of hybrid stability. Hybrid stability is affected by a number of factors, namely temperature, ionic strength, base composition, presence of formamide, hybrid length and the number of mismatched base pairs, each of which are discussed below. The type of hybrid formed also affects stability - RNA:RNA hybrids are more stable than the equivalent DNA:DNA hybrids (Wetmur et al,1981; Cox et al,1984).

The factors affecting hybrid stability have been related by the following equation for DNA:DNA hybrids (Thomas & Dancis, 1973):

$$T_m = 81.5 + 18.5(\log M) + 0.41(\%GC) - \frac{820}{L} - 0.6(\%F) - 1.4(\%m)$$

where M = monovalent cation concentration (mol/litre)

%GC = percentage G+C content of probe

L = probe length (bases)

%F = percentage formamide

%m = percentage mismatched base pairs.

RNA:RNA hybrids are approximately 10-15°C more stable than the equivalent DNA:DNA hybrid (Wetmur et al,1981; Cox et al,1984) and RNA:DNA hybrids are intermediate (Casey & Davidson,1977). The above equation relates to hybrids greater than 50 base pairs long. For probes less than 50 bases, such as oligonucleotides, the following equation has been derived for the formation of RNA:DNA hybrids (Fitzpatrick-McElligott et al,1988)

$$T_m = 81.5 + 16.6 \log M + 0.41(\%GC) - \frac{675}{L} - 0.65(\%F) - \%m.$$

However, the experimental determination of  $T_m$  for DNA:RNA and RNA:RNA hybrids formed *in situ* has been found to be decreased approximately 5°C compared to solution or filter hybridisation values (Brahic & Haase,1978; Cox et al,1984) and this is thought to be caused by fixation of the RNA in the tissue which may prevent the formation of full-length hybrids (Cox et al,1984).

The effect of each factor on hybrid stability is described below:

#### 1.2.1.1. Temperature.

Increases in temperature cause disruption of the hydrogen bonds that hold the hybrid together resulting in dissociation of the two strands.

#### 1.2.1.2. Ionic strength.

Increased ionic strength results in greater hybrid stability, due to neutralisation of the electrostatic forces between the negatively-charged phosphate

groups on opposite strands by the positive ions. High salt concentrations also result in decreased solubility of the bases, thus strengthening the hydrophobic interactions between them. Although divalent cations have a greater effect on hybridisation than monovalent ions, they are often co-factors for nucleases and so are avoided in hybridisation mixes.

#### 1.2.1.3. Base composition.

A higher GC content will increase hybrid stability, since three hydrogen bonds are formed in a GC base pair, compared to two for an AT pair.

#### 1.2.1.4. Formamide.

Both DNA and RNA are denatured when dissolved in aqueous formamide solutions. Formamide is used in preference to elevated temperatures for achieving high stringency hybridisations. This is because there is less degradation of nucleic acids at lower temperatures, increased retention of nucleic acids to filters or cells to slides, and better preservation of morphology for *in situ* hybridisation. In addition, there is no microbial growth in formamide solutions, even with extended incubation times at 37°C.

The  $T_m$  of DNA:DNA hybrids has been shown to decrease linearly with increased formamide concentrations (McConaughy et al, 1969) and reassociation of DNA occurs maximally at approximately  $T_m - 25^\circ\text{C}$  (Wetmur & Davidson, 1968). The effects of formamide on RNA:DNA hybrids are more complex. At high concentrations of formamide (>40%) there is maximal difference in stability between RNA:DNA and DNA:DNA hybrids, and at a temperature which is intermediate between the  $T_m$  for each hybrid, the RNA:DNA reaction is optimal whereas the DNA:DNA reassociation rates are negligible (Casey & Davidson, 1977):

#### 1.2.1.5. Hybrid length.

A longer probe will form a more stable hybrid than a short one because of the increased number of hydrogen bonds. However, very long probes are disadvantageous for *in situ* hybridisation due to problems in penetrating the cell. Base pair mismatches have a much greater effect on hybrid stability in short duplexes. For example, for DNA:DNA hybrids a reduction in  $T_m$  of approximately 5°C for each mismatch occurs in hybrids shorter than 20 base pairs (Wallace et al, 1981), compared to a reduction of 1°C per % base pair mismatch for hybrids greater than 150 base pairs (Bonner et al, 1973).

#### 1.2.2. Kinetics of hybridisation.

##### 1.2.2.1. Single-stranded probes.

In most hybridisation reactions, the probe is in vast excess over the target nucleic acid, so that the rate of formation of hybrids follows pseudo-first-order kinetics, i.e. amount of hybridisation is directly proportional to time until hybridisation is complete (Szabo et al, 1977). The time required for half of the probe to hybridise to an immobilised target is given by the equation (Meinkoth & Wahl, 1984):

$$t_{1/2} = \frac{N \cdot \ln 2}{3.5 \times 10^5} \times L^{0.5} \times C$$

where  $t_{1/2}$  is measured in seconds

and  $N$  = probe complexity (length of unique sequences in bases)

$L$  = probe length in bases

$C$  = probe concentration (mol.nucleotides/l)

The target nucleic acid concentration does not influence  $t_{1/2}$  when the probe is in excess. Ionic strength has little effect on hybridisation rate when maintained between



0.4M and 1.0M, and pH has little effect between pH5.0 and 9.15 (Wetmur & Davidson, 1968).

The kinetics of hybridisation of single-stranded RNA and DNA in solution and in filter hybridisations are very similar. However, the kinetics of *in situ* hybridisation differs from that predicted by the above equation and has been estimated to be approximately ten-fold lower (Szabo et al, 1977; Brahic & Haase, 1978; Haase et al, 1984a). This is due to decreased access of the probe to the target nucleic acid in the tissue.

#### 1.2.2.2. Double-stranded probes.

The kinetics of hybridisation using double-stranded probes are hard to determine. Since both strands are present in the hybridisation reaction, two competing reactions can occur - hybridisation between the probe and the target, and reannealing of complementary strands. Reannealing effectively reduces probe concentration thereby slowing the rate and amount of hybrid formation.

#### 1.2.2.3. Effect of dextran sulphate on rate of hybridisation.

The rate of hybridisation can be enhanced by the inclusion of anionic polymers such as dextran sulphate in solution and mixed phase hybridisations (Wahl et al, 1979) and in *in situ* hybridisation (Lederman et al, 1981). This is presumed to be due to exclusion of the probe from the volume occupied by the polymer, thus effectively increasing its concentration (Wahl et al, 1979). For single-stranded probes greater than 250 bases in length, the rate of hybridisation is increased approximately 20-30-fold for single-stranded RNA probes and 50-fold for single-stranded DNA probes (Lederman et al, 1981). No effect is seen for very short oligonucleotide probes (Meinkoth & Wahl, 1984). For double-stranded probes a significant increase in hybridisation rate of up to 100-fold is seen (Wahl et al, 1979). This additional increase is thought to be due to the formation of

"networks" of probe by the association of partially overlapping sequences, promoted by the presence of dextran sulphate (Lawrence & Singer, 1985).

### 1.3. METHODOLOGY OF *IN SITU* HYBRIDISATION.

A wide variety of methods have been described for *in situ* hybridisation. However, all methods follow five basic steps, outlined in Figure 1.1. The first and probably most important step involves fixation of the cells or tissue, to maintain cellular morphology and prevent loss of nucleic acids during subsequent steps. However, the extent of fixation should not be such that the probe is unable to penetrate the cells. Therefore pretreatment steps are frequently employed, which aim to reverse some of the effects of fixation by deproteinisation, thus rendering the cells more permeable to probe. Hybridisation of probe is carried out at relatively low stringency and follows the general principles of hybridisation described above. The use of factors such as formamide in the hybridisation solution, which increases the stringency of hybrid formation, allows the use of lower hybridisation temperatures than are commonly used for filter hybridisations, thus helping to preserve the morphological integrity of the tissue. Post-hybridisation treatments aim to remove unhybridised probe by extensive washing procedures and nuclease digestions. Finally, the presence of hybridised probe is visualised, either by autoradiography for radioactively labelled probes, or by colorimetric means for non-radioactively labelled probes.

### 1.4. CHOICE OF PROBE FOR *IN SITU* HYBRIDISATION.

Advances in cloning have resulted in the wide availability of both DNA and RNA probes for use in hybridisation studies. A number of factors affect the efficiency of a probe for hybridisation, such as probe length, base composition and whether it is single- or double-stranded. For *in situ* hybridisation, the advantages of increased probe length for greater hybrid stability must be weighed against the problems of



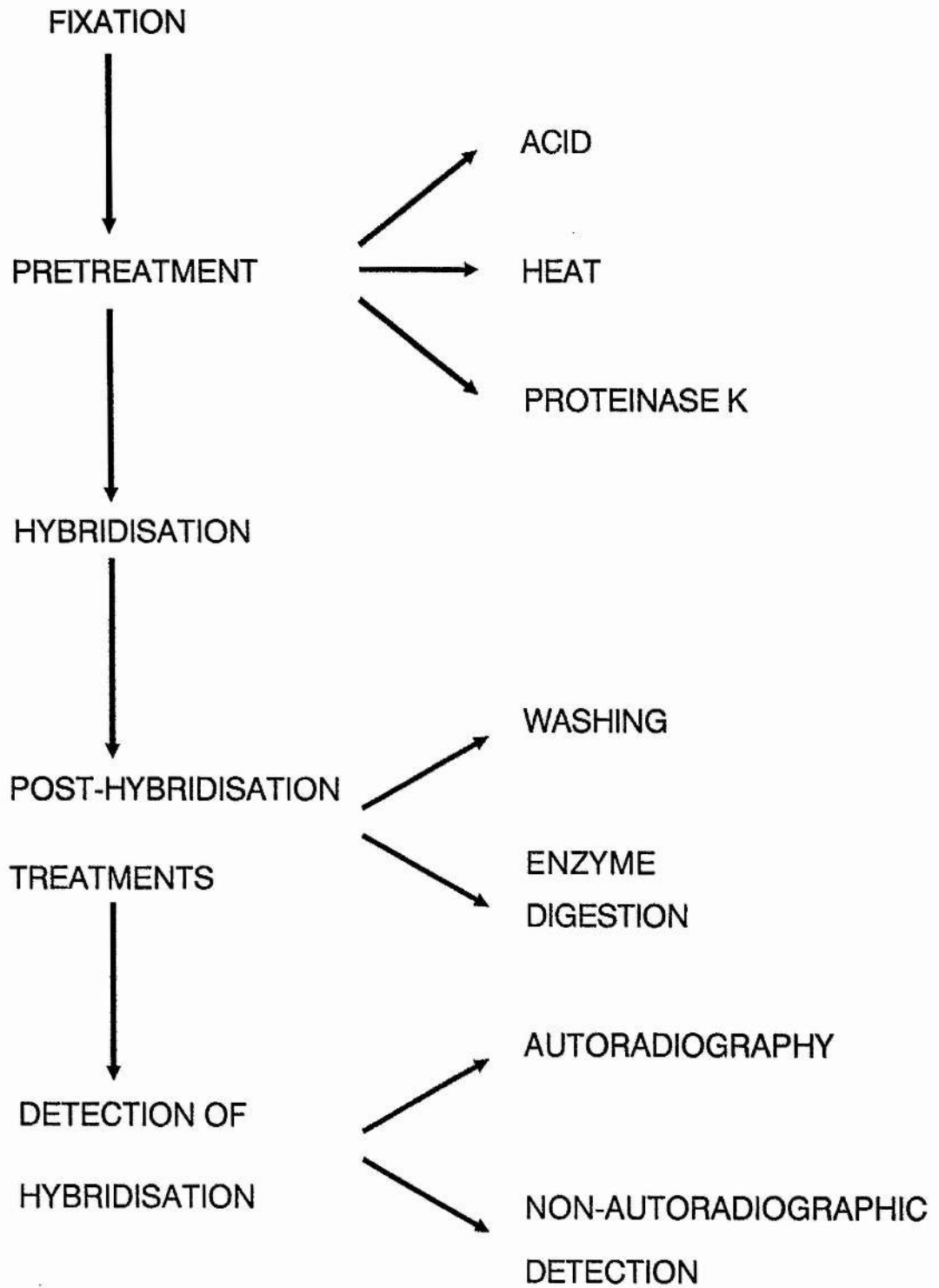


Figure 1.1. Outline of the methodology of *insitu* hybridisation.

cellular penetration of larger probes. Small probes such as oligonucleotides are ideal for penetration of cells but may form weak hybrids because of their size and are often less sensitive because of the limited amount of label that can be incorporated into them.

Double-stranded DNA probes labelled by nick-translation (Rigby et al,1977) have until recently been the most widely used type of probe in hybridisation studies including *in situ* hybridisation. However, there is now increasing use of single-stranded probes, particularly with the developments in oligonucleotide synthesis and the design of riboprobe vectors. The main advantage of double-stranded probes is that significant signal amplification is possible by network formation. There are however a number of disadvantages. Double-stranded probes require denaturation before addition to the hybridisation mix and reannealing of probe can occur, effectively removing probe available for hybridisation. Differential detection of hybridisation to strand-specific sequences is not possible and since vector sequences are also usually labelled, non-specific background binding is likely to be higher. Similarly, the higher probe concentrations required to overcome the effects of reannealing tend to result in higher backgrounds. Single-stranded probes therefore have a higher probing efficiency and generally give lower non-specific background levels. They also have the major advantage of being able to distinguish complementary sequences.

#### 1.5. CHOICE OF PROBE LABEL FOR *IN SITU* HYBRIDISATION.

The ideal label for *in situ* hybridisation should have both a high specific activity and a high resolution. However in practise, the choice of label is generally a compromise between the two, depending on requirements for sensitivity, resolution and speed.

### 1.5.1. Radioactive labels.

$^3\text{H}$  is the radioactive label of choice for *in situ* hybridisation for achieving high resolution since the track length of the  $\beta$ -particle in nuclear emulsion is only  $1\mu\text{m}$ . However, due to the low energy of the emissions long exposure times of 1 - 3 months are generally required for low frequency nucleic acid sequences.  $^{125}\text{I}$  can be used to give more rapid results with good resolution ( $1\text{-}10\mu\text{m}$ ) by the Auger electrons, although high background levels are sometimes seen. A potential advantage of  $^{125}\text{I}$  which has yet to be fully exploited is the use of the  $\gamma$ -rays to localise hybridisation to particular areas using X-ray film, then coating with nuclear emulsion to detect  $\beta$ -emissions within this area (Haase et al,1985a; Cunningham & Mundy,1987).  $^{35}\text{S}$  is usually the label of choice for *in situ* hybridisation in situations where resolution is less critical, such as the localisation of viruses within tissue. Resolution is in the range of  $10\text{-}15\mu\text{m}$ , and the higher energy emissions allow shorter exposure times of days to weeks. The inclusion of the reducing agent, dithiothreitol, can be used to help stabilise the radioactive molecule during storage of the probe. One disadvantage of  $^{35}\text{S}$  is that since the sulphur atom is foreign to the nucleotide, it is often incorporated less efficiently than  $^{32}\text{P}$  or  $^3\text{H}$  during probe labelling.  $^{32}\text{P}$ -labelled probes allow rapid detection of low copy number sequences but give poor resolution.  $^{32}\text{P}$  is therefore more often used for optimising conditions and for regional localisation over larger areas using X-ray film.

The physical properties of these labels are summarised in Table 1.1.

### 1.5.2. Non-radioactive labels.

The most commonly used non-radioactive label for hybridisation studies is biotin, which can be incorporated into the probe using biotin-labelled dUTP (Langer et al,1981; Singer & Ward,1982; Riley et al,1986), or chemically linked to the DNA or RNA (Al-Hakim & Hull,1988). Biotin can also be directly linked to the probe

Table 1.1. Physical properties of radioactive particles.

Isotope.	Half-life.	Emission.	Energy (keV).	Resolution. <sup>a</sup>
$^{32}\text{P}$	14.3 days	$\beta$	1.71	20-30um
$^{35}\text{S}$	87.4 days	$\beta$	0.167	10-15um
$^{125}\text{I}$	60 days	$\gamma$	0.035	-
		$\beta$	0.035	1-10um
$^3\text{H}$	12.35 years	$\beta$	0.018	0.5-1um

a. Scatter of electrons around a point source.

by the use of photobiotin, an analog of biotin, which is activated by irradiation with visible light to form a stable link with DNA or RNA (Forster et al,1985). Detection of hybridisation utilises either avidin or streptavidin which have a high affinity for biotin, or an anti-biotin antibody, linked to an enzyme for colorimetric detection. Other non-radioactive labels include digoxigenin, which is linked to dUTP and detected using a highly specific alkaline phosphatase-linked IgG (Baldino & Lewis,1989), and direct fluorescent detection of hybridisation using fluorochrome-labelled DNA or RNA (Bauman,1985).

The use of non-radioactive labels has a number of advantages over the use of radioactive ones. They provide a significantly increased resolution because the label is detected at the site of hybridisation, and with advances in immunogold labelling, resolution at the electron microscopic level is possible. In addition, they are non-hazardous and long exposure times are not required, therefore they have great potential for diagnostic use. The main drawback to their wide-spread use to date has been the reduced sensitivity compared to radioactive labels. However, new methods such as immunogold silver staining, are becoming increasingly more sensitive (Brigati et al,1983; Leary et al,1983; Burns et al,1985; Cubie & Norval,1989).

**CHAPTER TWO.**

**Labelling of single-stranded probes  
for *in situ* hybridisation studies.**

## 2.1. INTRODUCTION.

In this chapter, the types of single-stranded probes and labelling methods available for use with hybridisation studies are described. Three types of probe were selected for our studies, M13 DNA probes, oligonucleotide probes and RNA probes. The M13 and oligonucleotide probes were labelled by primer extension reactions and the RNA probes by *in vitro* transcription, and the efficiency of labelling of each probe type was compared.

### 2.1.1. Production of single-stranded M13 DNA probes.

M13 is a single-stranded bacteriophage which has been modified to form a number of vectors used for cloning. Single-stranded DNA is readily purified from phage particles released into the supernatant of infected cultures. The production of single-stranded probes from M13 clones has been developed from the method of dideoxy-sequencing, which utilises a unique primer which anneals to one side of a multiple cloning site (mcs) containing the insert of interest. This is extended using Klenow polymerase to incorporate labelled nucleotides and will be referred to as forward-labelling. Two methods have been used to label probes in this way:

#### 2.1.1.1. Forward-labelling.

Forward-labelling uses a universal primer which anneals to a region 3' to the mcs. Labelling of the insert sequence produces a partially double-stranded molecule (Figure 2.1.) which can then be cut by a unique restriction enzyme at the 5' side of the insert (Jeffreys et al, 1985). The single-stranded labelled probe is separated from the rest of the vector by denaturation and separation on an agarose gel. If low melting point (LMP) agarose is used, purification of the probe from the gel is not required and it can be used directly in the hybridisation mix. High specific activity probes are produced of  $10^8$ - $10^9$  dpm/ $\mu$ g.

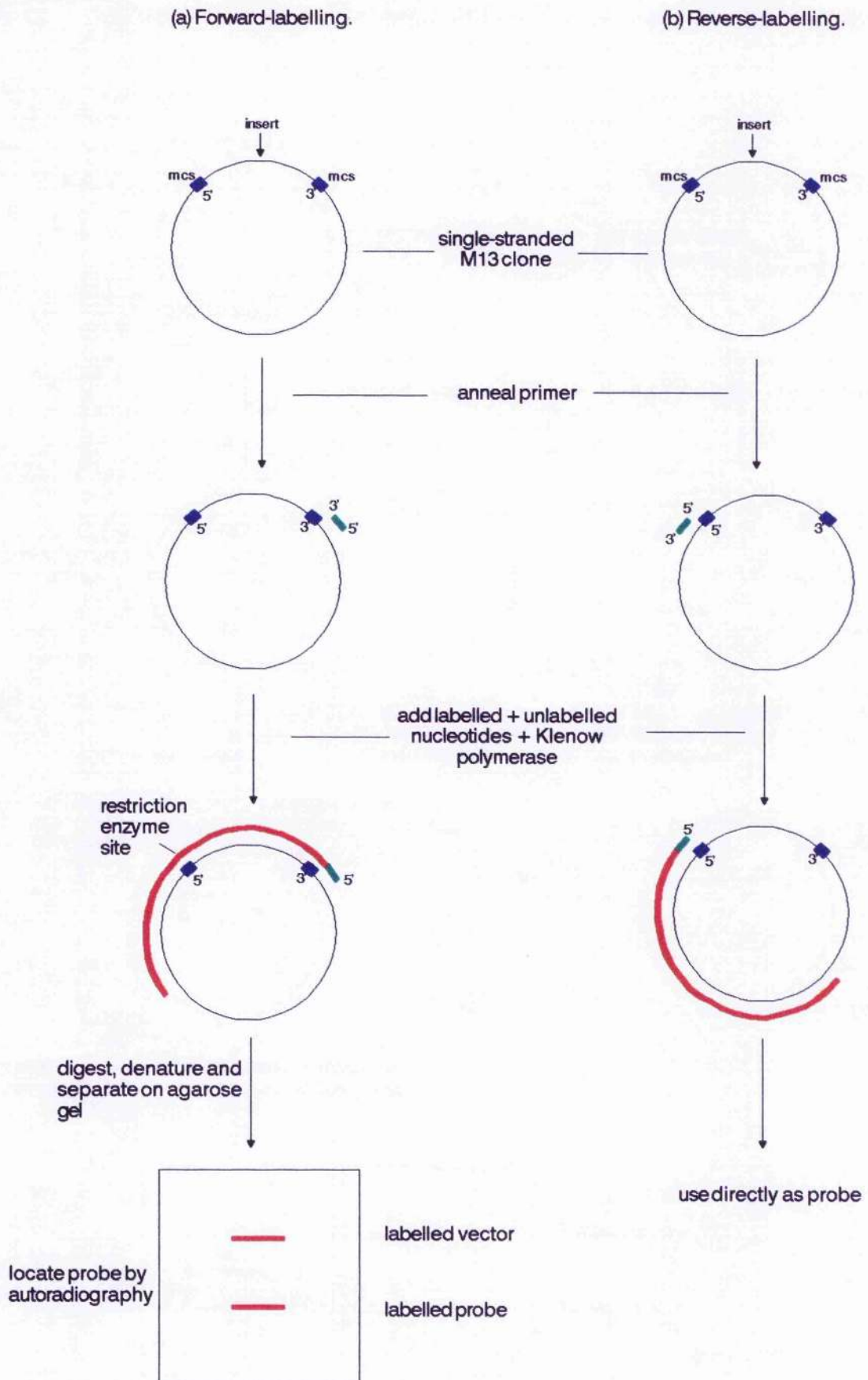


Figure 2.1. Production of single-stranded M13 DNA probes.



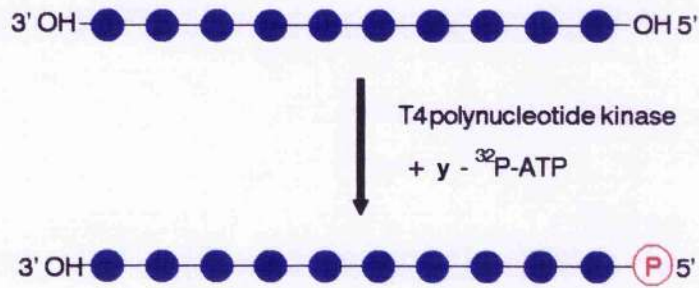
#### 2.1.1.2. Reverse-labelling.

This uses a primer which anneals to the 5' side of the mcs and therefore labels the vector sequences leaving the insert single-stranded to anneal during hybridisation (Figure 2.1.). To avoid readthrough into the insert, the reaction is limited by a low labelling nucleotide concentration. Probe denaturation is not required before use. High specific activities are attainable ( $10^8$ - $10^9$  dpm/ $\mu$ g) and since the vector is larger than the insert, amplification of the hybridisation signal is achieved relative to forward-labelled probes. However, the resulting large size of the probe is disadvantageous for *in situ* hybridisation due to difficulties in penetration of the cell.

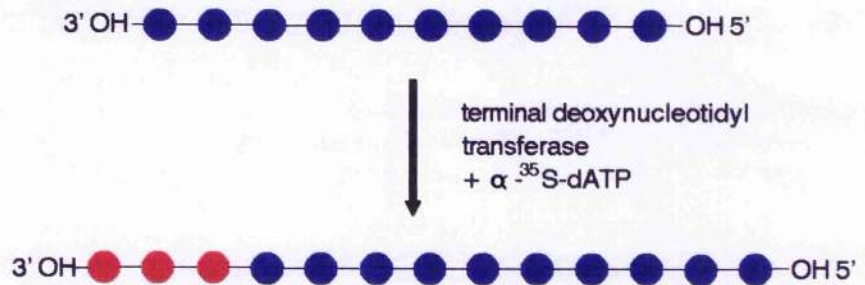
#### 2.1.2. Production of oligonucleotide probes.

With recent advances in automated DNA synthesis, short single-stranded DNA oligonucleotides of approximately 15-100 bases are becoming widely available for use as hybridisation probes. Access to an oligonucleotide synthesiser allows the rapid production of many probes from published sequence data, which can be designed to differentiate between highly homologous gene sequences. They are rapidly and easily made when compared to the time required for cloning procedures, although they may be expensive. Due to their small size they are ideal for penetration of cells for *in situ* hybridisation. Although high specific activity probes can be produced ( $10^8$ - $10^9$  dpm/ $\mu$ g), the sensitivity of oligonucleotide probes is lower than other types of probes because of their small size. Oligonucleotides may form weak hybrids but this can be partially overcome by selecting a sequence with a high G+C content where possible, to increase hybrid stability (Section 1.2.). Oligonucleotides can be labelled at either the 5' or 3' end, or by primer-extended synthesis of the opposite strand (Figure 2.2.).

(a) 5' end-labelling.



(b) 3' end-labelling.



(c) Primer extension.

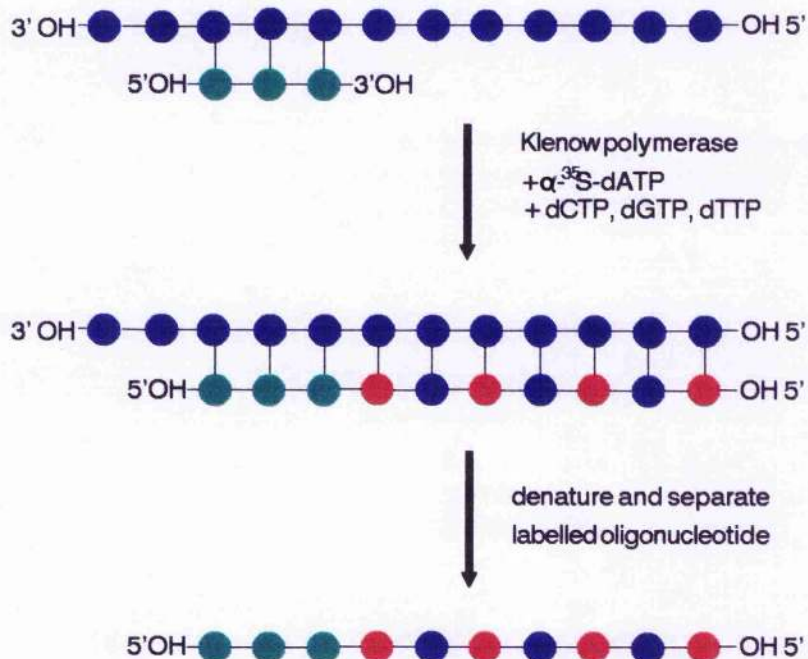


Figure 2.2. Labelling of oligonucleotide probes.

#### 2.1.2.1. 5' end-labelling.

Oligonucleotides are particularly well suited to this type of labelling since they are synthesised with a hydroxyl group in the 5' position in place of the normal phosphate group. Other DNA sources require dephosphorylation with alkaline phosphatase before labelling. The enzyme T4 polynucleotide kinase is used to catalyse transfer of the  $\gamma$ -phosphate group of ATP to the free 5'-OH group (Figure 2.2.(a)).  $^{32}\text{P}$ - $\gamma$ -ATP is generally used for this reaction although  $^{35}\text{S}$ - $\gamma$ -ATP is now available. However, only one molecule of isotope is added per probe molecule and thus specific activities are lower than those attainable by other labelling methods and cannot be increased (assuming all ends are labelled).

#### 2.1.2.2. 3' end-labelling.

The 3' end of an oligonucleotide can be labelled using the enzyme terminal deoxynucleotidyl transferase, which is template independent and continuously adds dNTPs to the 3' end forming a tail (Figure 2.2.(b)). The length of the tail can be controlled by time or deoxynucleotide concentrations. This method allows a wider choice of label ( $^3\text{H}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{125}\text{I}$  and biotin) than 5' end labelling since isotopes in the  $\alpha$  position are more readily available.

#### 2.1.2.3. Primer extension.

The method of primer extension using Klenow polymerase is similar to forward-labelling of M13 probes. A short oligonucleotide is synthesised which hybridises to a region at the 3' end of a template oligonucleotide and is extended to the end of the template incorporating labelled deoxynucleotides (Figure 2.2.(c)). The labelled and unlabelled strands are separated on a polyacrylamide gel and the probe band eluted. The template oligonucleotide synthesised must therefore be in the same sense as the sequence to which hybridisation is required, since the labelled probe

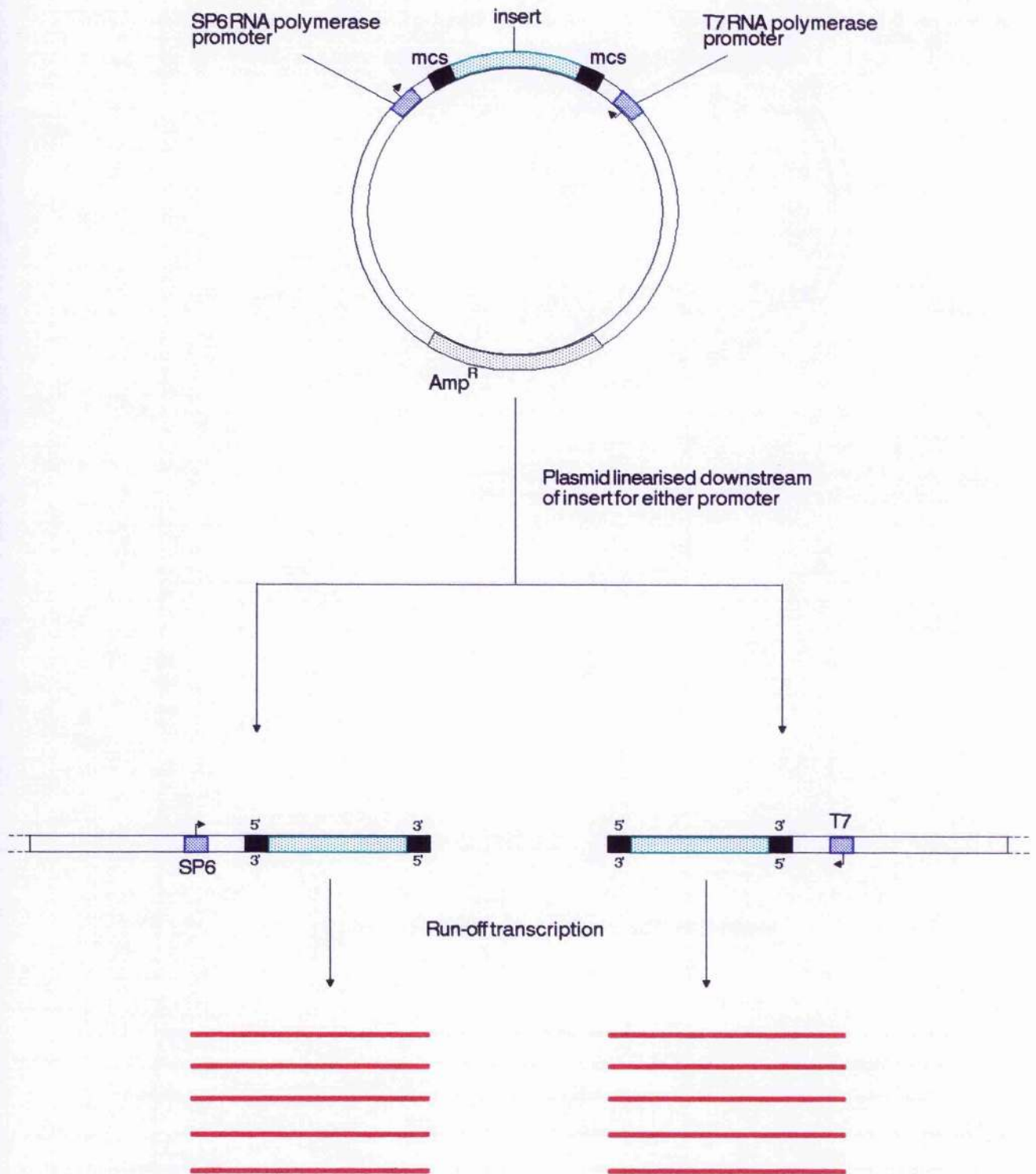


Figure 2.3. Production of single-stranded RNA probes by *in vitro* transcription.

will be complementary to the template. By selecting a primer which anneals leaving an overhang of a few bases at the 3' end, separation of the two strands is facilitated. However, the reaction must then be run on ice to inhibit the 3'-5' exonuclease activity of the Klenow polymerase which would remove this overhang. Labelling by this method allows high specific activities to be achieved ( $10^8$ - $10^9$  dpm/ $\mu$ g).

### 2.1.3. Production of single-stranded RNA probes.

The production of single-stranded RNA probes has recently been developed, using bacteriophage DNA-dependent RNA polymerases in conjunction with specifically designed plasmid transcription vectors into which the DNA sequence of interest can be cloned (Melton et al,1984; Mead et al,1986). The RNA polymerases from a number of bacteriophages, such as the *Salmonella* phage SP6 and the coliphages T7, T5 and T3 have a very high specificity for their own promoters so that the RNA produced is limited to the sequences cloned downstream of the polymerase promoter (Butler & Chamberlain,1982; Kassavetis et al,1982).

The most recently developed vectors contain two phage promoters in opposite orientations on either side of a multiple cloning site (Figure 2.3.). Linearisation of the vector at a site downstream of the insert prevents the transcription of vector sequences and defines the probe size. The two promoters allow transcripts of either sense to be produced. The DNA template is removed by digestion with DNase I, followed by ethanol precipitation or spin column purification of the labelled RNA, thus eliminating the need for electrophoretic purification. High specific activities of  $10^8$ - $10^9$  dpm/ $\mu$ g are attainable. The majority of transcripts (>80%) are full length, even when nucleotide concentrations are limiting (Melton et al,1984) which suggests that initiation is limited to a fraction of the template molecules, which are then elongated to full length transcripts.



## 2.2. MATERIALS AND METHODS.

### 2.2.1. Production of M13 probes.

A number of cloning vectors have been derived from M13, which utilise its natural replication cycle, allowing the isolation of single-stranded DNA from culture medium and double-stranded DNA from the bacterial cells. The most commonly used vectors are the **mp** vectors (Messing, 1983). These contain a fragment of the *E. coli* *lac* operon which includes the regulatory regions and the coding information for the first 146 amino acids of the  $\beta$ -galactosidase gene. This is able to complement a defective  $\beta$ -galactosidase gene on the F episome of the host cell, resulting in an active enzyme. A multiple cloning site (mcs) has been inserted into the amino terminal end of the M13  $\beta$ -galactosidase gene. This insertion does not affect the ability of the enzyme to complement the host cell  $\beta$ -galactosidase mutant. However, when DNA is cloned into the vector, the protein sequence is disrupted and complementation does not occur. When bacteria infected with M13 are plated on agar in the presence of the *lac* gene inducer IPTG and the chromogenic substrate X-gal, blue plaques are formed by cells which have undergone complementation. In cells infected with an M13 vector containing insert DNA, complementation cannot occur and the plaques remain colourless. This thus acts as a selectable marker for the presence of vector DNA containing an insert.

M13 clones containing inserts specific to the paramyxoviruses measles virus (MV) and canine distemper virus (CDV) were obtained from Dr. T. Barrett (Institute for Animal Health, Pirbright, Woking). An M13 clone for simian virus 5 (SV5) was prepared by J. Southern from a pBR322 clone of the HN gene obtained from Prof. R. Lamb (Northwestern University, Illinois). These probes are described in Table 2.1. Insert sequences were cloned into the *Sma*I site of the **mp8** vector or the *Pst*I site of the **mp9** vector, shown in Figure 2.4. Clones were obtained as

Table 2.1. Details of the M13 probes.

PROBE	VIRUS	GENE	SIZE	VECTOR	PROBE SENSE	CROSS-HYB <sup>b</sup>
Z26	MV	N	1kb <sup>a</sup>	mp8	m	-
59S	MV	N	1.4kb <sup>a</sup>	mp8	v	-
4	SV5	HN	1.5kb	mp9	m	NA
112H	CDV	F	ND	mp8	v	-
Y9	CDV	P	ND	mp8	m	+
Y12	CDV	P	ND	mp8	v	+

a. Size estimated on agarose gel.

b. Cross-hybridisation between MV and CDV.

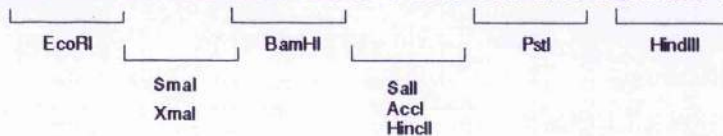
ND. Not determined.

NA. Not applicable.

M13mp8

(7244bp)

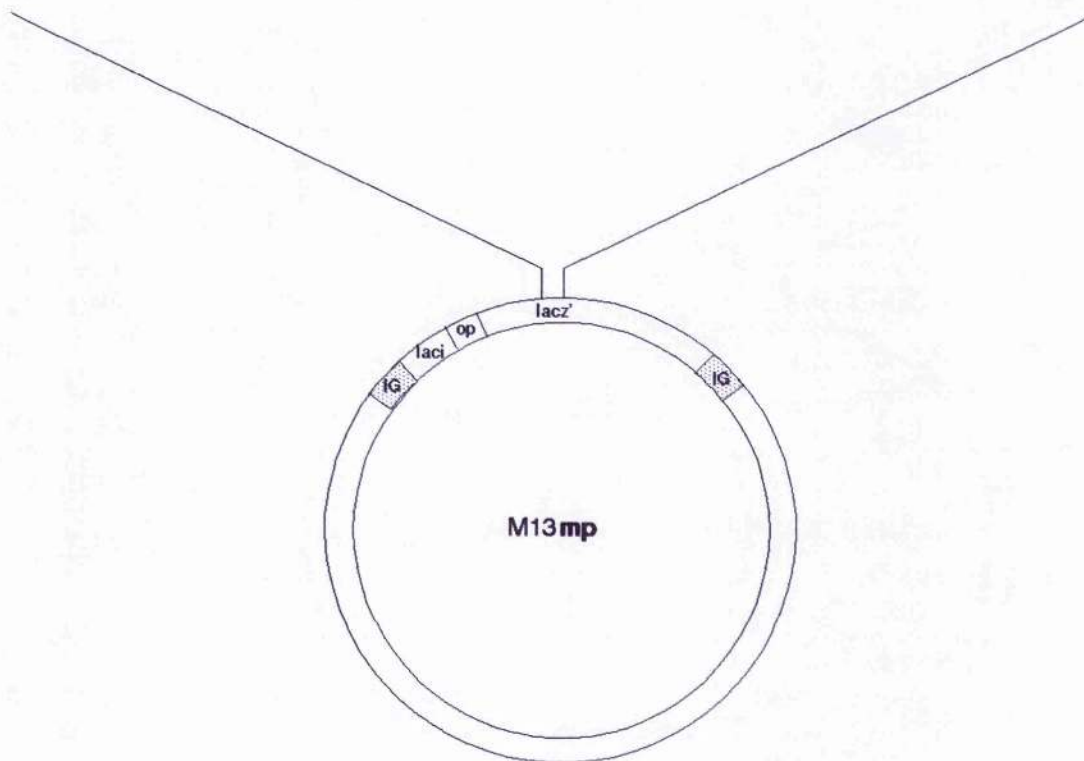
THR MET ILE THR ASN SER ARG GLY SER VAL ASP LEU GLN PRO SER LEU ALA LEU ALA  
 ATG ACC ATG ATT ACG AAT TCC CGG GGA TCC GTC GAC CTG CAG CCA AGC TTG GCA CTG GGC



M13mp9

(7244bp)

THR MET ILE THR PRO SER LEU ALA ALA GLY ARG ARG ILE PRO GLY ASN SER LEU ALA  
 ATG ACC ATG ATT ACG CCA AGC TTG GCT GCA GGT CGA CGG ATC CCC GGG AAT TCA CTG GCC



**Figure 2.4. Structure of the multiple cloning sites of the M13 mp8 and mp9 cloning vectors.**

Mp8 and mp9 vectors contain identical multiple cloning sites (mcs) inserted in opposite orientations, as shown. The *E. coli lacZ* gene encoding  $\beta$ -galactosidase, has been inserted into an intergenic (IG) region and the mcs inserted into the *lacZ* gene. The amino acid sequence of the N-terminus of  $\beta$ -galactosidase is shown in red.



single-stranded DNA and transformed into *E.coli* as described below. Single-stranded DNA was purified and radioactive probes produced by forward-labelling.

#### 2.2.1.1. Transformation of *E.coli* with M13 DNA.

Plasmid DNA can be artificially introduced into bacteria by the method of transformation. A number of methods have been described in which bacteria are rendered temporarily permeable to small DNA molecules. Since the process is inefficient and only a small proportion of the cells will stably maintain the plasmid, a selectable marker encoded by the plasmid is used to identify the bacteria which contain the new plasmid. For M13 vectors this involves complementation of the  $\beta$ -galactosidase gene as described above. For other plasmids, an antibiotic resistance marker is commonly used. The efficiency of transformation has been found to be increased by treating the cells with divalent cations, dimethylsulfoxide (DMSO), reducing agents such as dithiothreitol (DTT), and hexamine cobalt chloride, although the mechanism of action of these agents and the way in which DNA is taken up by the cells remains unknown. The method described below is adapted from that of Hanahan (1983).

##### (a) Preparation of competent cells.

*E. coli* (strain JM101) from a frozen stock were inoculated into 6.5mls of SOB (Appendix, Table II) and grown with vigorous shaking until the culture O.D. reached 0.4-0.6  $A_{600nm}$ . The cells were incubated on ice for 10 mins, pelleted at 2,500rpm in a bench centrifuge for 10 mins at 4°C, and the supernatant discarded. The cells were resuspended in 2mls TFB (Table II), incubated on ice for 10 mins and repelleted. The cells were resuspended in 1/125 of the original culture volume (0.5mls) of TFB and DMSO added to 3.5% (17.5 $\mu$ l). The cells were incubated on ice

for 5 mins, 17.5µl DMSO added and incubated again for 10 mins on ice. A further 17.5µl DMSO was added and the mixture incubated for 5 mins on ice. The cells are now said to be competent to uptake exogenous DNA. Competent cells were stored in aliquots at -70°C for up to 6 months.

**(b) Transformation of competent cells.**

An aliquot of 100µl of competent cells was placed in a pre-cooled tube and the required amount of DNA added (1-100ng). For optimal results, the volume of DNA should be less than 5% of the volume of competent cells. The mixture was incubated on ice for 30 mins, then the cells were heat shocked at 42°C for 90 secs and cooled on ice for 1 min. Four volumes of SOC (Appendix, Table II) were added and the cells incubated with gentle shaking at 37°C for 1 h to allow the cells to recover. The bacteria were then plated onto H agar plates in the presence of IPTG and X-gal, as described below.

**(c) Plaque assay for detection of transformation.**

Into pre-heated sterile bijoux bottles was added 4mls of melted H-top agar (Appendix, Table I), 25µl IPTG and 50µl X-gal (2% solution in DMF). The mixture of bacteria and phage was added to the agar solution, mixed gently and poured onto pre-warmed H agar plates (Table I). After allowing to set, the plates were inverted and incubated at 37°C overnight.

The presence of bacteria infected with M13 was indicated by turbid plaques in the bacterial lawn. M13 vectors containing an insert in the mcs gave rise to white (colourless) plaques, whereas those without an insert were blue. Clones containing inserts were selected and grown up by stabbing the culture with a sterile toothpick and inoculating into culture medium. M13-containing bacteria grown up in this way were stored at -70°C in 15% glycerol and used to inoculate cultures.

### 2.2.1.2. Preparation of single-stranded M13 DNA.

#### (a) Mini-preps.

The surface of a frozen culture of transfected JM101s, prepared as described above, was scraped with a sterile toothpick and inoculated into 1.5mls of an overnight culture of JM101s and grown at 37°C for at least 7 h with vigorous shaking. Following transfer to a 1.5ml Eppendorf tube, the cells were pelleted at 12,500rpm in a microcentrifuge for 5 mins and the supernatant transferred to a fresh tube. A solution of polyethylene glycol (PEG) was prepared by dissolving 1g of PEG in 2.5mls 5M NaCl and 0.5mls dH<sub>2</sub>O, and making up the volume to 5mls. PEG is a hydrophilic polymer which acts as an excluding precipitant by effectively forcing virus particles and proteins out of solution. M13 was precipitated from solution by the addition of 150µl of PEG solution. The mixture was gently mixed and left at room temperature for 10 mins until a precipitate could be seen. The virus was then pelleted by centrifugation at 12,500rpm in a microcentrifuge for 5 mins. The supernatant was discarded and the pellet respun to remove any excess supernatant. The pellet was then resuspended in 100µl M13 TE (Appendix, Table II), extracted twice with phenol to denature and remove viral proteins, and ethanol precipitated (Appendix, Section I). The concentration of DNA was measured using a spectrophotometer (Appendix, Section II).

#### (b) Maxi-prep.

Into 500mls of 2X TYE (Appendix, Table I) was inoculated 5mls of an overnight culture of JM101s and 0.25mls M13 supernatant. This was incubated overnight at 37°C with vigorous shaking. The bacteria were removed by centrifugation at 6,000rpm for 10 mins, and the supernatant transferred to a fresh tube. M13 was precipitated by the addition of 1/8 volume 40% PEG solution and 1/8

volume 5M NaCl, and left to stand at 4°C for approximately 15 mins until a "swirling" precipitate was seen. The phage was pelleted by centrifugation at 9,000rpm for 30 mins at 4°C, the supernatant discarded and the pellet respun to remove all PEG. The pellet was resuspended in 12mls of 20mM Tris-HCl pH8.0, 20mM NaCl, 1mM EDTA, and cesium chloride was added to a final concentration of 34% (w/v). The solution was placed in a 30ml heat seal tube, topped up with cesium chloride solution of the same density, and the tube sealed. The phage was centrifuged at 35,000rpm overnight at 25°C, and was visible as a bluish-white band, which was removed with a syringe. The cesium chloride was removed by dialysis against buffer for 2-3 h. Contaminating RNA was digested with 50µg/ml RNase A at 37°C for 1 h. M13 proteins were denatured by incubation in 0.5% sodium dodecyl sulphate (SDS) and 100µg/ml proteinase K at 55°C for 2 h, and removed by extraction with phenol, phenol:chloroform, chloroform and ether (Appendix, Section I). The purified M13 DNA was ethanol-precipitated and resuspended in M13 TE buffer.

#### 2.2.1.3. Production of single-stranded M13 probes by forward-labelling.

Purified single-stranded M13 DNA was dissolved to a concentration of 0.1mg/ml in M13 TE. (Appendix, Table III). To 4µl (400ng) was added to 3µl dH<sub>2</sub>O, incubated at 60°C for 5 mins to denature any secondary structure, then 1µl of 10X TM buffer (Table III) and 2µl M13 universal primer (4ng) was added and incubated at 60°C for 30 mins to allow the primer to anneal. Following a brief spin to remove condensation, the forward-labelling reaction was carried out as follows: 10µl CGT mix (Table III), 6µl M13 TE (Table III) and 3µl <sup>35</sup>S-dATP (Amersham, >1000Ci/mmol) were added to the M13-primer solution. Finally, 5U of Klenow polymerase were added, and the solution was incubated at 37°C for 15-30 mins, to allow incorporation of the radioactive nucleotide. 2.5µl 0.5mM dATP (excess) was

then added and incubated at 37°C for 15 minutes to ensure extension of all labelled DNA past the end of the mcs. The Klenow polymerase was heat denatured at 70°C for 10 mins (this step was optional) and the partially double-stranded DNA molecule digested with an appropriate restriction enzyme to cut at the 5' side of the mcs using EcoRI for mp8 vectors and HindIII for mp9 vectors. To achieve this, 4µl 10X enzyme buffer( Table III), 1.2µl 0.1M spermidine and 20U of enzyme were added and incubated at 37°C for 30 mins. The reaction was stopped by the addition of 5.2µl of 1.5M NaOH, 0.1M EDTA. Fifteen µl of 5X loading buffer (Table III) was added and the entire sample run on a 2% low melting point (LMP) agarose upright mini-gel at 100V for 30-45 mins until the bromophenol blue dye reached the bottom of the gel. The gel was removed from the apparatus, placed on a clean glass plate and exposed to β-max Hyperfilm (Amersham) for at least 1 h. The localised probe band was cut out, 100µl M13 TE buffer added, and the agarose melted at 80°C for 5 mins immediately prior to use. One µl of probe was counted in a scintillation counter to estimate the specific activity of the probe. Labelled probe was stored at -20°C for up to two months.

### 2.2.2. Production of oligonucleotide probes.

The method of solid phase oligonucleotide synthesis, in which the growing polynucleotide chain is attached to a solid support, has allowed the development of automated DNA synthesisers, since it removes the need to purify products from remaining unreacted reagents after each step. Instead they can simply be washed through the column. Following synthesis and cleavage of the oligonucleotide from the column, it can be analysed for purity, for example by end-labelling and electrophoresis. If necessary, it can be further purified to remove any shorter reaction products present. Desalting is often required prior to use of the oligonucleotide, to remove contaminating ammonium salts.

Oligonucleotide probes were designed specific for mRNA sequences of the paramyxoviruses MV, CDV and SV5. Sequences were chosen from published sequence information (Table 2.2.). Probes were labelled radioactively by the method of primer extension. Therefore template oligonucleotides were synthesised as described below, and short (8-mer) primers were designed to anneal 5-8 bases in from the 3' end, resulting in probes with a final length of 33 bases. Details of the template and primer sequences are shown in Figure 2.5.

#### 2.2.2.1. Synthesis of oligonucleotides.

Oligonucleotides were synthesised on an Applied Biosystems Model 381A DNA synthesiser, using  $\beta$ -cyanoethyl phosphoramidites. Beta-cyanoethyl phosphoramidites are chemically modified nucleotides. They have a di-isopropylamine group on the 3' phosphate moiety, a  $\beta$ -cyanoethyl protecting group on the 3' phosphorous and a dimethoxytrityl (DMTO) protecting group on the 5' hydroxyl (OH) (Figure 2.6.). Exposed amine groups on the bases are protected by addition of isobutyl groups.

Synthesis begins with the 3'-nucleoside phosphoramidite, which is attached to an insoluble matrix via an organic linker, and the appropriate reagents are added stepwise. Synthesis therefore occurs in the 3' to 5' direction. The DMTO protecting group must first be removed to allow addition of the incoming nucleotide to the 5' end of the chain. The incoming nucleotide is mixed with tetrazole during this coupling step, which protonates the nitrogen of the di-isopropyl group, allowing nucleophilic attack by the exposed 5'-OH group. This results in a 5'-3' linkage formed through a trivalent phosphorous group. A capping step follows every coupling step. This terminates or "caps" any oligonucleotide chains which failed to undergo addition, by acetylation. Following capping, the labile trivalent phosphorous linkage formed during the coupling step is converted to the stable pentavalent phosphorous linkage

Table 2.2. Details of the paramyxovirus oligonucleotide probes.

PROBE	VIRUS	GENE	SIZE	SENSE	%G+C	SP.ACT <sup>a</sup>	%HOMOLOGY	NUCLEOTIDES	REFERENCE
228924	MV	NP	33b	V	58	$1.1 \times 10^9$	45 <sup>b</sup>	353-390	Rozenblatt et al, 1985
ABO35	SV5	HN	33b	V	67	$8.0 \times 10^8$	-	523-562	Hiebert et al, 1985
ABO30	CDV	NP	33b	V	58	$1.2 \times 10^9$	45 <sup>c</sup>	353-393	Rozenblatt et al, 1985

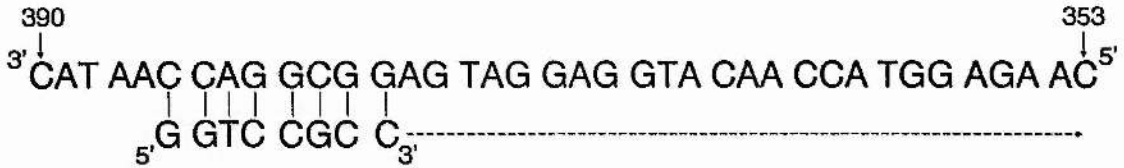
a. cpm/ug (labelled by primer extension with <sup>35</sup>S-dCTP (650 Ci/mmol)).

b. Homology with CDV.

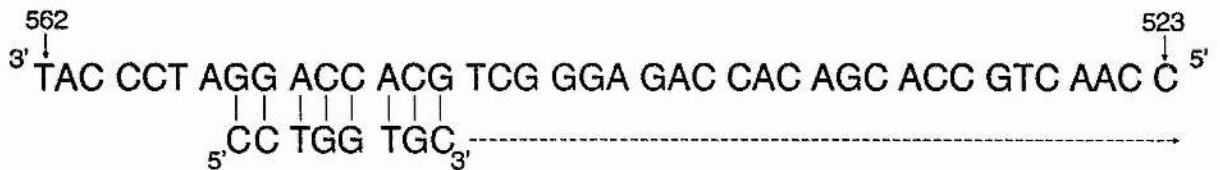
c. Homology with MV.



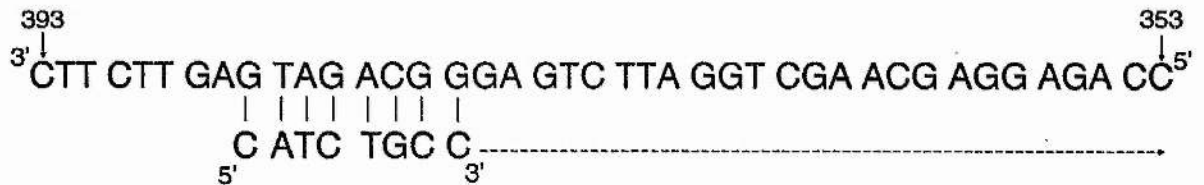
(a) MV (228924).



(a) SV5 (ABO35).



(c) CDV (ABO30).



**Figure 2.5. Sequences of the paramyxovirus oligonucleotide templates and primers.**

Oligonucleotide sequences were selected from published sequence data (see Table 2.2.). Probes were labelled by primer extension of the primer as shown. Template sequences were synthesised as message sense so that the resulting probes hybridised to mRNA. Primer sequences were chosen to hybridise 5-8 bases in from the end of the template, to facilitate separation of the two strands after labelling. Final probe length was 33 bases for all probes.



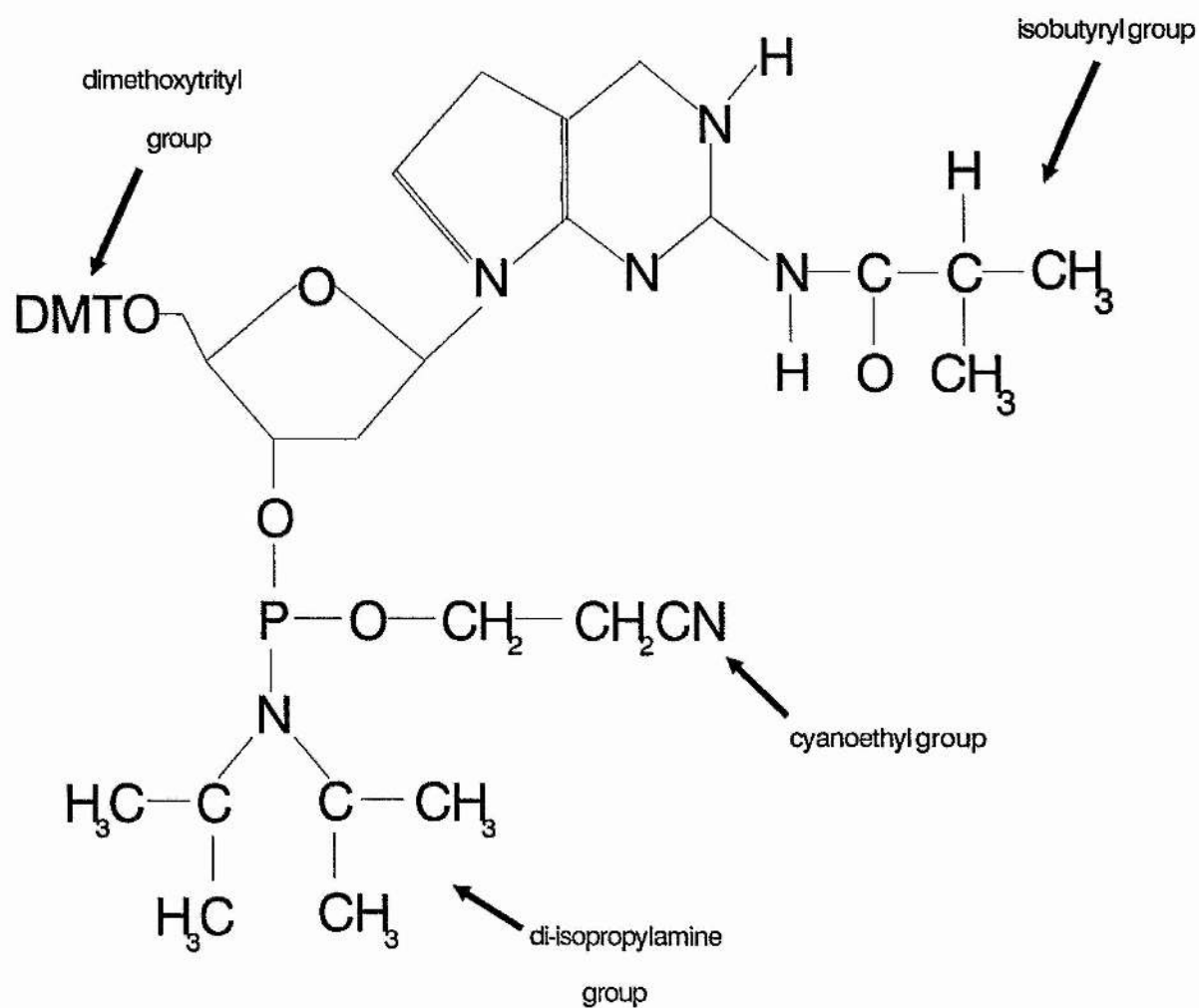


Figure 2.6. Structure of guanosine cyanoethyl phosphoramidite.

present in DNA, by oxidation with iodine. The cycle is repeated until the required oligonucleotide has been synthesised.

#### 2.2.2.2. Cleavage and deprotection.

After synthesis was complete, the column was washed with concentrated ammonium hydroxide, which cleaved the oligonucleotide chains from the solid support. This was done by inserting a syringe at both ends of the column and washing four times for 15 mins each, with a total volume of 3.5mls ammonium hydroxide, which was finally collected in one syringe. The base protecting  $\beta$ -cyanoethyl groups were removed by incubation at 55°C overnight. The ammonium hydroxide was then removed by evaporation under vacuum, and the DNA pellet was redissolved in water. For oligonucleotides less than 15 bases in length, the DNA was dried down and resuspended in water twice more to remove some of the ammonium salts. Complete desalting was carried out using a Biogel column as described below. Further purification of short oligonucleotides was not generally carried out. For oligonucleotides greater than 15 bases in length, the DNA was ethanol-precipitated with 3 volumes of ethanol, 0.1 volumes 3M sodium acetate, and magnesium acetate was added to 10mM to enhance precipitation of low molecular weight DNA. The pellet was washed with 70% ethanol, dried and resuspended in water. The concentration was determined by measuring absorbance at 260nm on a spectrophotometer. The purity was analysed by 5' end-labelling and polyacrylamide gel electrophoresis. Larger oligonucleotides were then generally further purified by polyacrylamide gel electrophoresis (see below).

#### 2.2.2.3. Desalting.

The crude oligonucleotide sample in ammonium hydroxide contains various organic salts resulting from the deprotection steps. Desalting may therefore be

required before the sample is used in enzymatic reactions, if it is not purified by gel electrophoresis. Desalting was carried out by size exclusion chromatography using a 5ml Biogel P6 column equilibrated with TEN buffer (Appendix, Table IV). The entire oligonucleotide sample in a volume of approximately 100 $\mu$ l was loaded onto the column with bromophenol blue marker dye. Fractions of 200 $\mu$ l were collected until the dye reached the bottom of the column and the absorbance at 260nm of each fraction was determined to identify the oligonucleotide peak and measure its concentration.

#### 2.2.2.4. 5' end-labelling of oligonucleotides.

Radioactive labelling of the 5' end of the oligonucleotide was used to analyse the proportion of shorter reaction products present in the mixture. The enzyme T4 polynucleotide kinase was used to catalyse the phosphorylation of the 5'OH from  $\gamma$ -<sup>32</sup>P-ATP. Approximately 150ng of oligonucleotide (in 1-2 $\mu$ l) was added to 1 $\mu$ l 10X kinase buffer (10mM MgCl<sub>2</sub>, 10mM Tris-HCl, 10mM EDTA, 10mM BSA), 2.4 $\mu$ l  $\gamma$ -<sup>32</sup>P-ATP (Amersham; 3000Ci/mmol) and 10U polynucleotide kinase. The volume was made up to 10 $\mu$ l with dH<sub>2</sub>O and incubated at 37°C for 1 h. The reaction was stopped by the addition of 20 $\mu$ l TEN buffer and 20 $\mu$ l loading buffer, and the entire sample was loaded onto a 5ml column of Biogel P6 to separate labelled oligonucleotide from unincorporated label. Fractions of approximately 200 $\mu$ l were collected and the samples within the first radioactive peak (containing the oligonucleotide) were pooled. For analysis of purity, a sample of labelled oligonucleotide was run on a 10% polyacrylamide/7M urea sequencing gel (0.4mm thick) and exposed to X-ray film overnight. If a significant proportion of the oligonucleotide preparation was less than full length, it was further purified as described below.

#### 2.2.2.5. Purification of oligonucleotides by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis was used to remove shorter reaction products from the crude oligonucleotide sample. A relatively large amount of DNA needed to be applied to the gel to enable visualisation by UV shadowing. A thicker gel (1.5mm) was therefore used to accommodate the larger sample.

Oligonucleotide (100-200 $\mu$ g) in a volume of 100 $\mu$ l was added to 100 $\mu$ l deionised formamide and EDTA to a final concentration of 10mM. The sample was loaded on a 1.5mm 10% polyacrylamide/7M urea gel and electrophoresed until the BPB dye had run approximately 2/3 of the length of the plates. The dye was loaded in a separate well to avoid contamination of the sample and masking of the UV shadow. The gel was removed from the plates and placed on clingfilm on top of a fluorescent thin layer chromatography plate. The UV-absorbing oligonucleotide appeared as a shadow when the plate was viewed under short-wave UV light (240-300nm). The highest molecular weight band was cut out, the gel crushed and the oligonucleotide eluted overnight at 37°C in 1ml of 0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA and 0.1% SDS. The supernatant was passed through a glass wool filter to remove any polyacrylamide, and the gel washed once with 0.5ml buffer, which was added to the rest of the supernatant. The volume was reduced to 100-200 $\mu$ l by water extraction with butan-1-ol and the sample desalted as described previously. The concentration of DNA was measured on a spectrophotometer.

#### 2.2.2.6. Primer-extension labelling of oligonucleotide probes.

The labelling reaction was carried out as follows: 30ng template DNA and 10ng primer were added to 1 $\mu$ l each of dATP, dGTP and dTTP (1mM stocks) and 1.5 $\mu$ l 10X reaction buffer (100mM Tris-HCl pH8.5, 100mM MgCl<sub>2</sub>). Three  $\mu$ l <sup>35</sup>S-dCTP (Amersham, >1000Ci/mmol) was added, and lastly 5U of Klenow polymerase. The

volume was made up to 15µl with dH<sub>2</sub>O and incubated on ice for 2 h (to inhibit the 3'-5' exonuclease activity of Klenow which would remove the template overhang). The reaction was stopped by the addition of an equal volume of deionised formamide and denatured at 65°C for 3-4 mins to separate the two strands immediately before loading onto the gel. The sample was run on a 0.5mm 10% polyacrylamide/7M urea gel. The BPB marker dye was loaded separately and the gel run until the dye had travelled at least 2/3 the length of the gel. The upper plate was removed and the gel covered with clingfilm and exposed to X-ray film for at least 1 h to localise the labelled oligonucleotide. The probe band was cut out and eluted in 250µl 1mM EDTA by shaking at 37°C for at least 2 h. The supernatant was recovered and a sample counted on a spectrophotometer to determine the specific activity of the probe.

### **2.2.3. Production of RNA probes.**

Two measles virus-specific clones (MVN, MVH) were obtained from Dr L. Cosby, Queens University, Belfast. The clones had been constructed in Gemini vectors (Promega) described in Figure 2.7. and contained inserts specific to the nucleoprotein gene (clone MVN) and the haemagglutinin gene (clone MVH) of measles virus (Figure 2.7.; Table 2.3.). The plasmids used contained both SP6 and T7 promoters, allowing transcripts of either sense to be produced.

The clones were obtained as plasmid DNA and transfected into *E.coli* (strain JM101). Plasmid DNA was purified from culture and radioactive probes produced by *in vitro* transcription as described below.

#### **2.2.3.1. Transformation of *E.coli*.**

Riboprobe vectors were obtained as plasmid DNA and transformed into *E.coli* (strain JM101) as previously described for M13 DNA, except that following

Table 2.3. Details of the MV RNA probes.

Probe	Gene	Size	Vector	Sp. Act. (cpm/ug) <sup>a</sup>	Nucleotides
MVN	NP	183bases	pGEM-blue	$2.4 \times 10^8$	1374-1557
MVH	H	312bases	pGEM-1	$3 \times 10^8$	8146-8458

a. Labelled with <sup>35</sup>S-UTP (1250 Ci/mmol).

**Figure 2.7. Structure of the riboprobe plasmids and measles virus clones.**

(a) MVN clone.

The Sau 3A fragment of the MV nucleoprotein (N) gene (183bp; nucleotides 1375-1558) was inserted into the BamHI site of the riboprobe system vector pSP64 (Melton et al,1984). The insert was then cut out with EcoRI/HindIII and inserted into the EcoRI/HindIII sites of the Gemini system vector pGem-Blue, to form the cloning plasmid shown on the following page. The resulting plasmid therefore has the multiple cloning site (mcs) of pSP64 as shown. The structure of the MVN clone is shown.

Linearisation with EcoRI and transcription with SP6 polymerase resulted in negative sense probes which hybridise to mRNA. Linearisation with HindIII and transcription with T7 polymerase resulted in positive (message) sense probes which hybridise to viral RNA.

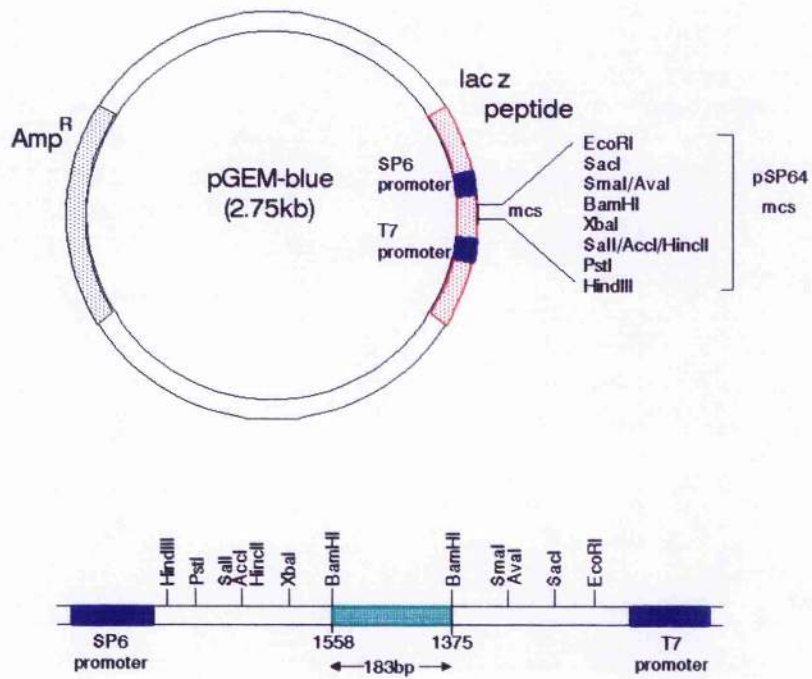
(b)MVH clone.

The Aval/SacI fragment of the MV haemagglutinin (H) gene (312bp; nucleotides 8145-8457) was inserted into the Aval/SacI sites of the riboprobe Gemini 1 vector (pGem-1) as shown on the following page.

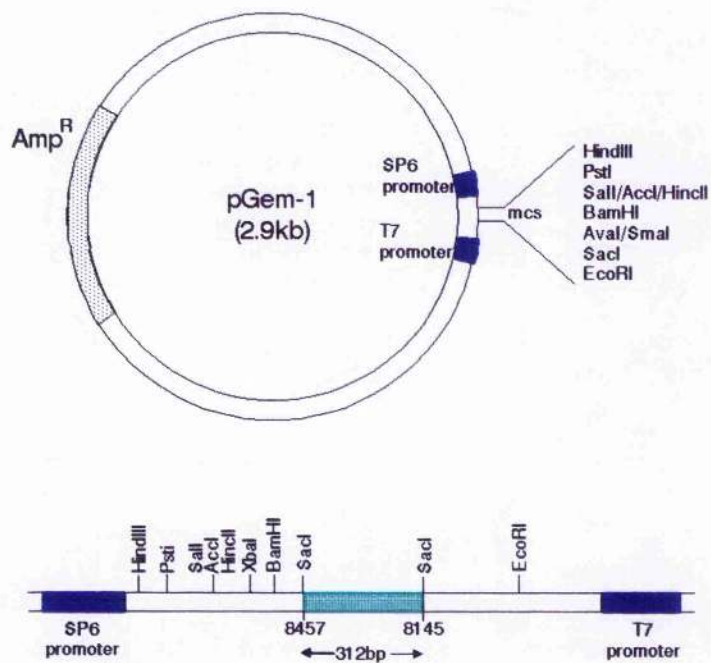
Linearisation with EcoRI and transcription with SP6 polymerase results in negative sense probes which hybridise to mRNA. Linearisation with HindIII and transcription with T7 polymerase resulted in positive sense probes which hybridise to viral RNA.



(a)



(b)





heat-shocking at 42°C and incubation on ice, 10 volumes of Luria broth (Appendix, Table I) containing ampicillin at 50µg/ml was added and the cells were grown at 37°C for 1 h to allow expression of the ampicillin gene. The cells were concentrated by brief centrifugation and resuspended in 100µl Luria plus ampicillin, and plated out on Luria plus ampicillin plates. Only cells containing the ampicillin-resistance gene should grow to form colonies. A single colony was picked, grown up and plasmid DNA purified.

#### 2.2.3.2. Plasmid purification.

Plasmid DNA which replicates at high copy number can be readily purified from bacterial cultures grown in the presence of the appropriate antibiotic. Separation of plasmid DNA from bacterial DNA relies on the fact that plasmid DNA is generally much smaller than bacterial DNA and exists as a covalently closed circle, whereas the bulk of bacterial DNA extracted is obtained as broken linear molecules. It is therefore important to avoid the action of nucleases so that the circular plasmid form is maintained, and hence EDTA is included in all steps.

The Gram negative cell wall of *E.coli* is first lysed with lysozyme and EDTA. The outer cell membrane is disrupted by EDTA, allowing the lysozyme to attack the cell wall and is carried out in the presence of glucose to prevent immediate osmotic lysis of the spheroplasts formed. The spheroplasts are then lysed by the addition of detergent (SDS) and the bacterial DNA is denatured with alkali (pH 12.0-12.5). Covalently-closed circular DNA is denatured at this pH but rapidly reanneals. When the cell extract is then neutralised by the addition of acid and salt, the chromosomal DNA is precipitated, most probably because reassociation of long single-stranded molecules occurs at multiple sites to form an insoluble mass. Most cellular RNA is also precipitated. Denatured proteins, DNA and RNA are removed by centrifugation and the plasmid DNA in solution is concentrated by propanol

precipitation, which preferentially precipitates larger fragments of DNA. The plasmid DNA can then be further purified from any contaminating bacterial DNA by cesium-chloride density gradient centrifugation by using the intercalating dye ethidium bromide. Covalently-closed circular DNA binds less ethidium bromide than linear DNA due to physical constraints on the molecule, and hence will have a greater buoyant density when centrifuged to equilibrium, forming a band below the chromosomal DNA. The detailed protocol for plasmid purification is described below.

An overnight culture of JM101s was grown up from a single colony in 10mls Luria broth at 37°C with shaking. A volume of this culture (0.25mls) was inoculated into 100mls of Luria broth supplemented with ampicillin at a concentration of 50µg/ml and grown with shaking at 37°C until the O.D.<sub>600nm</sub> reached 0.4-0.6 A (late log phase). One litre of Luria broth plus ampicillin was inoculated with 50mls of this culture and shaken at 37°C overnight. The bacteria were pelleted at 6,000rpm for 10 mins, washed by resuspension in STE buffer (Appendix, Table IV), repelleted and thoroughly resuspended in 20mls Solution I (Table IV). They were allowed to stand at room temperature for 5 mins, 40mls of Solution II (Table IV) was added and gently mixed, and the mixture was incubated on ice for 10 mins. Thirty mls of Solution III (Table IV) was added and the solution incubated on ice for a further 10 mins. The cellular debris was removed by centrifugation at 20,000rpm for 20 mins at 4°C and the supernatant was transferred to a fresh tube. Isopropanol (0.6 volumes) was added and stood at room temperature for 15 mins, and the plasmid DNA was pelleted at 10,000rpm for 30 mins at room temperature. The pellet was washed in 70% ethanol, briefly respun, and dried under vacuum. The DNA was resuspended in 2mls TE buffer (Table IV), and the final volume was measured. 1.2g of cesium chloride and 50µl ethidium bromide (10mg/ml stock) were added per ml of DNA. This was mixed thoroughly until the cesium chloride was dissolved, then spun in a microcentrifuge for 5 mins to remove

debris formed by ethidium bromide and bacterial protein complexes. The supernatant was loaded into a 2ml Beckman Quickseal tube and topped up with a solution of identical cesium chloride and ethidium bromide concentration. The plasmid DNA was banded by centrifugation in a Beckman benchtop ultracentrifuge at 80,000rpm overnight or at 100K for 4 h. The lower plasmid DNA band was harvested by puncturing the tube and withdrawing the DNA into a syringe. The cesium chloride was removed by passing over a G50 Sephadex column equilibrated with TEN buffer (Table IV) and collecting 200µl fractions. This also separated the DNA-bound ethidium bromide from free ethidium bromide. The first DNA-containing peak was detected under UV light and pooled. Ethidium bromide was removed by ion-exchange chromatography over a 1ml Dowex AG50 column prepared in a pasteur pipette and equilibrated with STE buffer (Table IV). The sample was applied to the column and the eluate collected. The column was washed with 1ml STE buffer, the eluate collected and pooled with the first sample. The DNA was then ethanol precipitated, pelleted at 10,000rpm for 15 mins at 4°C, washed in 70% ethanol, dried and resuspended in 1ml TE buffer. The purity of the plasmid was assessed by running a sample on a 0.6% agarose mini-gel containing 0.5µg/ml ethidium bromide and viewing under UV light. Contaminating RNA was removed by digestion with 50µg/ml RNase A at 37°C for 1 h, followed by phenol extraction and ethanol precipitation. The DNA was resuspended in TE buffer and the concentration measured using a spectrophotometer.

#### 2.2.3.3. Labelling of RNA probes.

##### (a) Preparation of DEPC-treated water.

Diethyl pyrocarbonate (DEPC) is an irreversible inhibitor of RNases. DEPC-treated water was prepared by making a 0.2% solution and then autoclaving to remove the DEPC. To avoid RNase contamination, all solutions were made up with DEPC-treated water, Eppendorfs were washed in DEPC-treated water and autoclaved,

and gloves were worn at all times.

(b) Linearisation of plasmid.

Approximately 20µg of plasmid DNA was cut with an appropriate restriction enzyme (Figure 2.7.) at the 3' end of the insert, then extracted once with phenol:chloroform, twice with chloroform, once with ether, and ethanol precipitated. The pellet was resuspended in DEPC-treated water, the concentration measured and 1µg analysed on a 0.6% mini-agarose gel containing 0.5µg/ml ethidium bromide, next to a sample of uncut plasmid to check for complete cutting.

(c) In vitro transcription.

*In vitro* transcription was carried out as follows: solutions were added to an Eppendorf tube in the following order - 3µl DEPC-treated water, 10µl 10X transcription buffer (Appendix, Table V), 5µl 0.1M DTT, 1µl 0.1M spermidine, 1µl 5mg/ml BSA (RNase-free), 2µl RNasin (Promega, 30U/µl), 10µl 5X XTP mix (Table V), 10µl 5X α-S-UTP (NEN, Table V), 1µl linearised plasmid (1µg), 5µl <sup>35</sup>S-UTP (Amersham; >1000 Ci/mmol) and 20U SP6 or T7 RNA polymerase (Promega). The solution was incubated at 40°C for 1 h. The template DNA was then removed by the addition of 2µl RNase-free DNase (23U/µl) and incubated at 37°C for 15 mins. The reaction was stopped by the addition of 150µl DEPC-treated water and the labelled RNA separated from unincorporated label and digested template by spin column purification. The specific activity of the probe was estimated by counting a sample in a scintillation counter.

## 2.3. RESULTS.

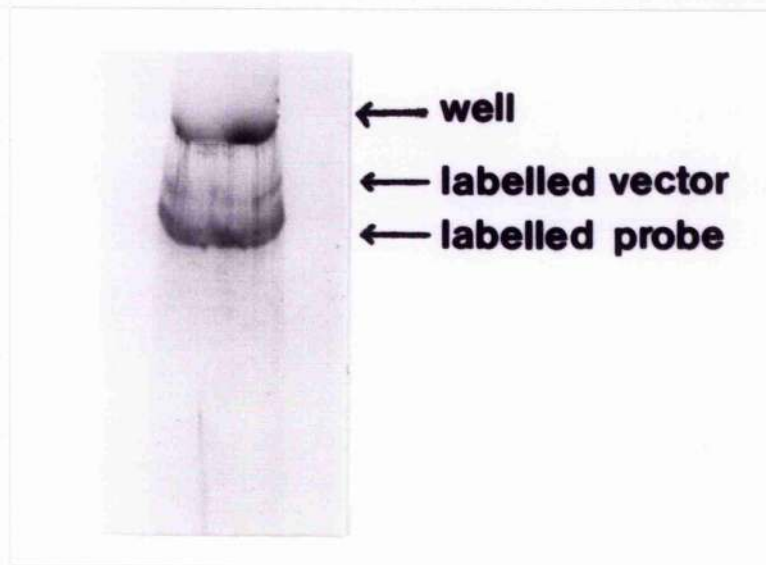
### 2.3.1. Labelling of M13 DNA probes.

Single-stranded DNA probes specific to the paramyxoviruses MV, CDV and SV5 were produced by forward-labelling of M13 clones, with the incorporation of  $^{35}\text{S}$ -dCTP. Separation of labelled probe DNA from labelled and unlabelled vector sequences was carried out by electrophoresis on a 2% low melting point (LMP) vertical agarose mini-gel and the probe band located by autoradiography (Figure 2.8.). The use of a vertical gel allowed more efficient detection of radioactivity as the labelled DNA was nearer to the surface of the gel compared to the use of a horizontal gel and was therefore not absorbed by the gel. Autoradiography was carried out using the wet gel covered with clingfilm, and the probe band was cut out of the gel and melted immediately prior to dilution in hybridisation solution.

The labelling reaction was chased with excess cold dCTP to ensure that all cDNA copies had been extended past the end of the multiple cloning site, thus allowing efficient restriction enzyme digestion. Specific activities therefore could not be accurately calculated, since the proportion of radioactive label in the probe was not known. Rough estimates were made by assuming a G+C content of 40% and that 50% of the cytosine residues were radioactively labelled. Specific activities calculated in this way were approximately  $5 \times 10^8$  cpm/ $\mu\text{g}$ , and between 1 and 15ng of probe were generally produced per labelling reaction. Although measurements calculated in this way are likely to be inaccurate, it allowed some degree of standardisation of probe concentrations for subsequent experiments.

There was considerable variation in the efficiency of labelling by this method. This appeared to be caused by decomposition of the buffer solutions used for labelling, since replacement with fresh solutions resulted in restoration of labelling efficiency. It was not affected by the purity of the DNA, since variable labelling occurred with both mini- and maxi-preparations of DNA.





**Figure 2.8. Purification of M13 probes by electrophoresis and autoradiography.**

M13 probes produced by forward-labelling with  $^{35}\text{S}$ -dCTP were separated from unlabelled and labelled vector sequences by denaturation and separation on a 2% low melting point agarose gel. After autoradiographic localisation as shown, the probe band was cut out and melted immediately before use. Shown above is purification of the M13 probe 4 which contains an insert of 1.5kb specific to the SV5 HN gene.

### 2.3.2. Labelling of oligonucleotide probes.

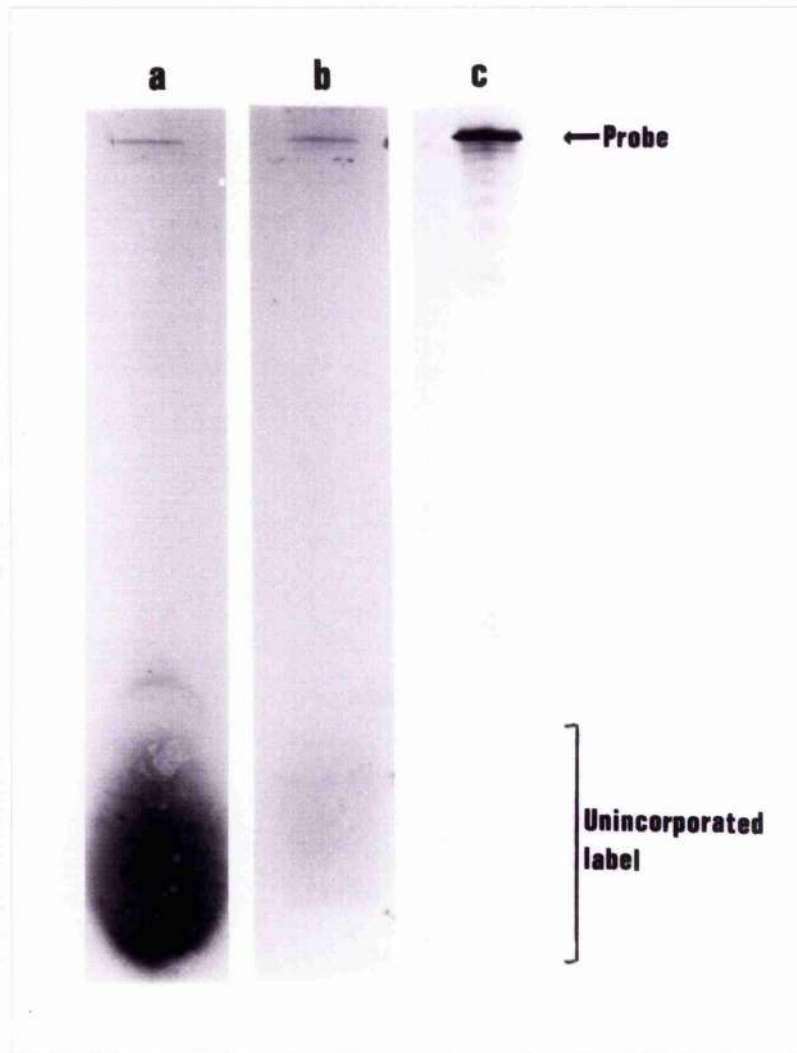
Oligonucleotide probes specific to MV, CDV and SV5 were labelled by primer extension with incorporation of  $^{35}\text{S}$ -dCTP. This nucleotide was chosen because it gave the highest specific activity for the probe sequences used. Specific activities could be accurately calculated because there was no incorporation of cold dCTP and ranged from  $8.0 \times 10^8$  to  $1.2 \times 10^9$  cpm/ $\mu\text{g}$  (Table 2.2.) The amount of probe produced per labelling reaction was approximately 5-10ng (from 30ng template).

Templates were synthesised in the message sense so that the resulting probe was viral sense and hence hybridised to mRNA. All probes produced were 33 bases in length. The labelled and unlabelled oligonucleotides were separated on a 10% polyacrylamide/7M urea gel and the labelled probe located by autoradiography. A single probe band was obtained and shorter reaction products were never seen (not shown).

Electrophoretic purification of the template oligonucleotide from incomplete products of the synthesis prior to labelling was found to significantly increase the efficiency of labelling, even when these shorter products appeared to comprise only a minor fraction of the template DNA, as determined by 5' end-labelling or UV shadowing.

### 2.3.3. Labelling of RNA probes.

Labelling of RNA probes was carried out by transcription *in vitro*, following linearisation of the plasmid DNA template. The transcription reaction was monitored by electrophoresis of a sample of the probe on a 10% polyacrylamide/7M urea gel, followed by autoradiography. A single full length probe band was produced with shorter species never comprising more than a minor proportion of the labelled RNA (Figure 2.9.). Unincorporated nucleotides were removed efficiently by spin column purification (Figure 2.9., compare lanes (a) and (b)). The transcription reaction was stimulated by the addition of spermidine and BSA (Figure 2.11., lane (c)), as



**Figure 2.9. *In vitro* transcription of riboprobes.**

Single-stranded RNA probes were produced by *in vitro* transcription. The labelling reaction was monitored by electrophoresis of a sample of the labelled probe (1/20 of total probe) on a 10% polyacrylamide/ 7M urea gel and exposure to X-ray film.

(a) Labelled probe prior to spin column purification.

(b) Labelled probe after spin column purification.

(c) Purified labelled probe transcribed with addition of BSA and spermidine.



previously reported by Melton et al (1984). The labelling reaction included cold  $\alpha$ -S-UTP at a ratio of 4:1 (cold:hot) which was incorporated with the same efficiency as  $^{35}\text{S}$ -UTP. Specific activities could therefore be accurately calculated and were approximately  $2\text{--}3 \times 10^8$  cpm/ $\mu\text{g}$ . Decreasing the amount of cold UTP in order to increase the specific activity resulted in limiting concentrations of UTP and shorter transcription products (not shown).

RNase contamination frequently occurred during labelling, despite treatment of all solutions with DEPC, the use of RNasin in the labelling reaction and the use of gloves at all times. The presence of RNase in the probe preparation was indicated by breakdown of probe when incubated overnight at  $37^\circ\text{C}$  and analysed on a polyacrylamide gel as described previously (not shown). This contaminating RNase activity was inhibited by the addition of SDS to a final concentration of 0.1% (not shown).

A comparison of the methods of labelling of DNA and RNA probes is summarised in Table 2.4.

Table 2.4. Comparison of the production of M13, oligonucleotide and RNA probes.

Probe	Average size	Length of labelling procedure	Electrophoretic purification	Input template DNA	Total probe produced	cpm/ $\mu$ g	cpm/pmole
M13	1-1.5 kb	1 day	yes	400ng	1-15ng	$5 \times 10^8$	$1 \times 10^7$
Oligo	33b	1-2 days	yes	30ng	5-10ng	$1 \times 10^9$	$9 \times 10^8$
RNA	200-300b	2 hours	no	1 $\mu$ g	50-100ng	$3 \times 10^8$	$3 \times 10^7$

## 2.4. DISCUSSION.

Three types of single-stranded probes selected for use in *in situ* hybridisation studies were compared with respect to ease of preparation and efficiency of labelling. Although earlier *in situ* hybridisation studies relied mainly on the use of double-stranded DNA probes labelled by nick-translation (Rigby et al,1977), or more recently, random priming (Feinberg & Vogelstein,1983), there has been increasing use of single-stranded probes, both DNA and RNA, particularly with advances in oligonucleotide synthesis and the development of riboprobe cloning vectors.

Although double-stranded probes have the advantage that they can provide an amplified hybridisation signal due to the formation of networks, they have a number of disadvantages compared to single-stranded probes for hybridisation studies. They require denaturation prior to hybridisation and the presence of both complementary strands in the hybridisation reaction results in the effective removal of much available probe by reannealing of the two strands. Hence larger amounts of probe are required than for single-stranded probes in order to achieve the same effective concentration. Single-stranded probes thus have a higher probing efficiency and also tend to result in lower levels of non-specific background hybridisation, since a lower probe concentration is required and there are no labelled vector sequences which might bind non-specifically. In addition, a major advantage of single-stranded probes is the ability to distinguish between complementary sequences.

In Chapter Three, experiments were undertaken to optimise conditions for the detection of viral RNA by using cells infected with the single-stranded negative sense paramyxoviruses. The choice of single-stranded probes thus allowed a distinction to be made between detection of viral RNA and the complementary mRNA. The three types of probes chosen were M13 DNA probes, oligonucleotide probes and RNA probes.

Production of M13 probes by the method of forward-labelling (Jeffreys et al,1985) was the least consistent of the three labelling methods used for probe production. Although electrophoretic separation of labelled probe from labelled and unlabelled vector sequences was required, the large difference in size enabled rapid separation on a mini-agarose gel. The use of low melting point agarose allowed direct use of the probe for hybridisation, without the need for further purification steps.

There are only a few examples of the use of M13 probes for filter hybridisation studies (Ricca et al,1982; Biggin et al,1984; Jeffreys et al,1985) or *in situ* hybridisation (Haase et al,1985b; Berger,1986). This is presumably a reflection on the need to clone into a specialised vector. However, the widespread use of M13 clones for sequencing studies should be able to provide a source of "ready-made" probes, by collaboration with appropriate research teams.

With recent advances in oligonucleotide synthesis and the wider availability of oligonucleotide synthesisers, oligonucleotide probes are now commonly used for hybridisation studies and are particularly suitable for *in situ* hybridisation because of their small size, allowing easy penetration of cells (Uhl et al,1985; Berger,1986; Lewis et al,1986; Cohen et al,1988; Guitteny et al,1988). The reduced hybrid stability of such small probes means that oligonucleotides can be used to distinguish between highly homologous sequences and can detect point mutations in both DNA (Wallace et al,1979; Bos et al,1984; Studenki & Wallace,1984) and RNA (Buvoli et al,1987).

Oligonucleotides can be labelled at either the 5' or 3' end or by extension of a primer. High specific activity probes produced by 3' tailing with  $^{32}\text{P}$  have been found to be comparable in sensitivity to double-stranded nick-translated probes for filter hybridisations (Collins & Hunsaker, 1985). Although the resolution with  $^{32}\text{P}$ -labelled probes is generally inadequate for *in situ* hybridisation, 5' end-labelling with  $^{32}\text{P}$ , where the incorporation of only one radioactive nucleotide per probe molecule results in a lower specific activity than 3' tailing, can give

adequate cellular resolution (Lewis et al,1986; Cogan et al, 1987).  $^{35}\text{S}$  is more usually the label of choice for *in situ* hybridisation with oligonucleotide probes. Although  $\gamma\text{-}^{35}\text{S}\text{-ATP}$  has recently become available (Amersham) for 5' end-labelling, the resulting specific activity will be low. Therefore 3' tailing is more commonly used (Guitteny et al,1988).

In these experiments, the method of primer extension (Studenki & Wallace,1984) with  $^{35}\text{S}$ -labelled nucleotides was chosen to label oligonucleotide probes. By selecting the appropriate nucleotide for maximum labelling, probes of high specific activity were produced. This method also allowed maintenance of a constant probe length for comparisons of small and large probes. This is not achieved by 3' tailing where it may be difficult to standardise the length of the tail. The method of primer extension labelling has been successfully used for *in situ* hybridisation with both  $^{35}\text{S}$ - and  $^3\text{H}$ -labelled oligonucleotides (Uhl et al,1985; Berger,1986). Primer extension labelling was found to work consistently. The major disadvantage of the method was the requirement for the high resolving power of a polyacrylamide gel to separate the labelled probe from unlabelled template differing by only a few nucleotides in length. In addition, further purification of the probe from the gel was required which was time-consuming.

Production of high specific activity RNA probes by transcription *in vitro* was found to be the simplest and most productive method of probe labelling, with a total reaction time of 1-2 hours. The main advantage came from the ability to remove the template plasmid DNA by digestion, thus avoiding the need for electrophoretic separation. In addition, large amounts of full-length probe were produced with relatively efficient incorporation of label (20-40% compared to 1-10% for M13 and oligonucleotide probes).

The development of the riboprobe transcription vectors (Melton et al,1984; Mead et al,1986) relies on the unusually high specificity of the RNA polymerases of

the bacteriophages SP6, T7 and T3 for their own promoters (Butler & Chamberlain,1982; Kassavetis et al,1982) so that only DNA cloned downstream of the promoter is transcribed. This thus allows the production of large amounts of specific RNA sequences. Single-stranded RNA probes are reported to give increased hybridisation sensitivity over double-stranded probes for both filter hybridisations (Brahic & Haase,1978; Diaz et al,1981; Zinn et al,1983; Church & Gilbert,1984; Meinkoth & Wahl,1984; Melton et al,1984) and *in situ* hybridisation (Cox et al,1984). Although no comparisons have been made with single-stranded DNA probes, RNA probes would be expected to provide a better signal:noise ratio because of their increased hybrid stability (Wetmur et al,1981). Although higher levels of non-specific hybridisation often occur with RNA probes, this can be effectively removed by digestion with RNase which selectively digests single-stranded RNA but not RNA that forms part of a hybrid. A disadvantage of the use of RNA probes is the need to subclone into specialised vectors. The need to avoid RNase contamination also makes them more problematic to use.

In conclusion, of the three labelling methods chosen for production of single-stranded probes for *in situ* hybridisation studies, the production of RNA probes by *in vitro* transcription was the most rapid and efficient method, producing large amounts of probe with relatively efficient incorporation of label. Labelling of oligonucleotide probes by primer extension was consistent but involved time-consuming purification steps. Production of M13 probes by forward-labelling was the least consistent of the three methods.

**CHAPTER THREE.**  
**Optimisation of conditions for**  
***in situ* hybridisation.**

### 3.1. INTRODUCTION.

The aim of the experiments described in this section was to optimise hybridisation conditions *in situ* for detection of viral RNA, using the three types of single-stranded probes labelled in Chapter Two. Optimisation of hybridisation conditions was investigated using an *in vitro* tissue culture system involving infection of cell monolayers with various paramyxoviruses. This system allowed reproducibility of experiments and direct comparison of results.

Although it is necessary to determine hybridisation conditions experimentally, an understanding of the principles involved can act as a guideline for initial choice of appropriate conditions. Each step in the technique of *in situ* hybridisation has therefore been described in some detail below. Optimal hybridisation conditions were determined separately for DNA and RNA probes.

#### 3.1.1. Fixation.

Fixation is probably the most important step for *in situ* hybridisation since it must fix the nucleic acids in the cell and maintain cellular structure whilst still allowing the probe to penetrate. The essential function of any fixative is to stabilise the protein structure of the cell and hence maintain morphology during subsequent treatments. A fixative should therefore be able to penetrate rapidly and react quickly to inactivate autolytic enzymes before they can cause damage to the cells. Ideally the fixative should not distort cellular structure. However, in practice, most fixatives cause either swelling or shrinkage to varying degrees (Figure 3.1.).

Fixatives can be divided into two groups, defined by their reaction with soluble protein (Baker, 1960) - coagulant (e.g. ethanol, methanol, acetone) and non-coagulant or cross-linking fixatives (e.g. formaldehyde, glutaraldehyde).



### 3.1.1.1. Coagulating fixatives.

Coagulating fixatives transform the cellular protoplasm (cytoplasm and nuclear sap) into a microscopical spongework. Proteins contain various hydrophilic groups, such as the  $\text{-NH}_2$  and  $\text{-COOH}$  groups of basic and acidic amino acids, the  $\text{-OH}$  of tyrosine and the  $\text{-C=O}$  group of the protein backbone. Under normal conditions, proteins interact with water via these groups. During fixation by coagulating fixatives, these interactions are disturbed and the protein becomes denatured. Ethanol, methanol and acetone are dehydrating agents and are thought to compete with water, removing it from its interaction with the active protein groups, which are thus left free to react with each other. They can form bonds close enough to exclude much of the water that previously lay between the protein chains, leading to coagulation. The interfaces formed between the "dry" protein and the surrounding fluid scatter light forming the white coagulum seen after fixation.

Nucleic acids are precipitated but not fixed by these fixatives and nucleoprotein is not coagulated. All three fixatives cause shrinkage of the cells, with acetone causing the greatest amount followed by ethanol (Figure 3.1.). Morphology is generally inferior to that with cross-linking fixatives and a coarse coagulum is produced in the cytoplasm and nucleus. Since the nucleoprotein is not stabilised in position, it may eventually be lost from the cell, therefore these are poor fixatives for nuclei or chromosomes. Since nucleic acids are not fixed, RNA may also be lost from the cell during subsequent treatments. However, many of these disadvantages can be overcome by the use of ethanol and methanol in conjunction with acetic acid. Acetic acid is an anomalous fixative in that it does not fix ordinary proteins. It reacts only with nucleoproteins when the  $\text{pH} < 4$ , causing separation of the DNA and protein and resulting in precipitation. When the  $\text{pH} > 4$ , there is no fixation and the tissue macerates. Acetic acid alone causes massive swelling of the cells to 4-5 times their original volume (Figure 3.1.). The acid is thought to break links between protein

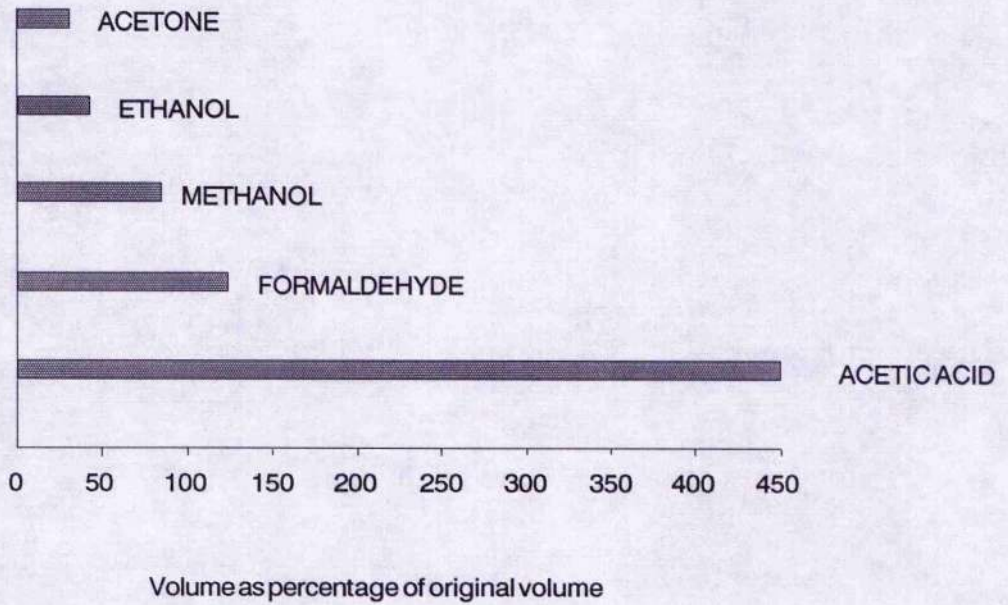


Figure 3.1. Graph showing the changes in volume undergone by gelatin-albumin gels in various fixatives (adapted from Baker, 1958).

chains, leading to exposure of hydrophilic groups and an influx of water. Thus when ethanol or methanol are combined with acetic acid (usually in a ratio of 3:1, ethanol:acetic acid) each compensates for the disadvantages of the other - the shrinkage caused by ethanol is offset by the swelling action of acetic acid, and the nucleoproteins which are unfixed by ethanol are stabilised by acetic acid.

### 3.1.1.2. Non-coagulating fixatives.

Formaldehyde and glutaraldehyde are cross-linking fixatives which react with amine groups, for example on lysine and glutamine residues. The cross-links formed cause formation of a gel-like structure. Cross-linking generally results in brittleness, loss of elasticity, reduction in ability to bind water and decreased solubility of proteins.

Formaldehyde (HCHO) reacts with proteins to form methylene bridges between amine groups, for example between lysine and glutamine residues. Formaldehyde also reacts with amine groups of both nucleic acids and nucleoproteins - it can penetrate through the protein shell due to its small size and cross-link the nucleic acids, thus preserving the conformation of nucleoproteins. Cellular morphology is generally well preserved. In paraffin-embedded sections, considerable distortion may occur, but good results are obtained with frozen tissue. Formaldehyde is frequently used with an 'indifferent' or non-fixative salt, which improves fixation although the mechanism is not understood. It is also helpful to add sucrose, for as yet unexplained reasons, particularly for electron microscopy.

Glutaraldehyde is a 5-carbon dialdehyde ( $\text{CHO}(\text{CH}_2)_3\text{CHO}$ ). It is the most effective of the aldehydes for preserving fine structure. Proteins are not denatured to any great extent and appear to retain their tertiary structure although biological activity is usually lost. Glutaraldehyde cross-links proteins by reaction with amine groups, by a mechanism similar to formaldehyde. However, the exact nature of the

reaction is more complex, due to the presence of numerous polymerised forms of glutaraldehyde in solution. There is some retention of nucleic acids but it is not known whether they are fixed or held in position by cross-linking of the proteins.

#### 3.1.1.3. Fixatives for *in situ* hybridisation.

There are widely conflicting reports of fixation conditions best suited to *in situ* hybridisation. Optimum fixation requires a compromise between preservation of morphology and efficient probe penetration. Certain fixatives will therefore be more applicable than others depending on the requirements for good morphology, retention of nucleic acids etc. A number of comparative studies have been carried out but the conclusions reached are often conflicting, emphasising the need to determine appropriate conditions experimentally.

#### 3.1.2. Pretreatment of cells.

Various deproteinisation steps are used to increase permeability of the tissue to the probe. The need for such pretreatment varies, depending on the type of fixative used, type of tissue, probe length, etc. Typical treatments include incubation in dilute acid, digestion with proteases, and high temperature incubations. Pretreatment must be optimised since excessive treatments may result in loss of RNA from the cell and loss of morphology.

#### 3.1.3. Hybridisation.

The hybridisation solution contains components intended to saturate sites in the tissue which bind probe non-specifically and hence cause non-specific background signals. These typically include bovine serum albumin, polyvinyl pyrrolidone and ficoll to decrease binding to proteins, and nucleic acids and EDTA to decrease non-specific nucleic acid interactions. Dextran sulphate is included to increase the



rate and/or intensity of signal of hybridisation, and formamide to reduce the temperature required for optimum hybridisation.

Important factors to consider when optimising the hybridisation step are probe length and concentration, stringency of conditions with respect to hybrid formation and kinetics, and preservation of morphology. Hybridisation is usually carried out at a relatively low stringency temperature of  $T_m - 25^\circ\text{C}$  for maximum rate of hybridisation (see section 1.2.) and is carried out in the dark to minimise ionisation of formamide. The optimum probe concentration which gives the maximum signal-to-noise ratio, must be determined experimentally. Background noise increases linearly with probe concentration (Cox et al, 1984), so the optimum concentration will be the lowest required to saturate the target nucleic acid sequences.

#### **3.1.4. Post-hybridisation treatments.**

Since hybridisation is carried out under low stringency conditions, non-specific binding of probe may occur. Post-hybridisation treatments decrease the amount of non-specific binding by washing the samples with increasing stringency, and in some cases, by enzyme treatments. The stringency of washing can be increased by altering any of the factors that affect hybrid stability such as salt concentration, temperature or formamide, to dissociate weakly complementary hybrids (see Section 1.2.).

RNA probes tend to give higher levels of non-specific binding than DNA probes. However, this can be selectively removed by treatment with RNase, which digests single-stranded RNA but not RNA which is part of a hybrid. Post-hybridisation enzyme treatments are not generally carried out for DNA probes, although S1 nuclease which acts on single-stranded DNA has been applied (Godard, 1983; Haase & Stowring, 1977).

### 3.1.5. Reduction of background noise.

Non-specific binding of probe can be a problem for *in situ* hybridisation since favourable signal:noise ratios must be achieved for detection of low level signals.

Non-specific hybridisation signals can arise in various ways:

- (i) Formation of weakly complementary hybrids.
- (ii) Electrostatic interactions with charged groups, such as positively charged proteins.
- (iii) Physical trapping of the probe within the cell.
- (iv) Artifacts of the detection system

Imperfect duplexes are removed by increasing the stringency of washing procedures. Electrostatic interactions between phosphate groups of the probe and basic proteins in the tissue can often be reduced by acetylation of the cells (Hayashi et al, 1978) and extensive washing steps are used to ensure that no unhybridised probe remains trapped in the tissue. Detection systems can sometimes give false positives. In liquid emulsion techniques, overdrying of the emulsion can cause stress, leading to the formation of silver grains. When detecting biotin-labelled probes with a peroxidase system, care must be taken that endogenous peroxidases in the tissue are inactivated.

### 3.1.6. Detection of hybridisation by autoradiography.

#### 3.1.6.1. Principles of autoradiography.

Autoradiographic emulsions consist of a suspension of silver bromide crystals in a gelatin matrix. The effect of light or radioactivity on a silver bromide crystal is to excite the outer electron of the bromide ion out of its orbit, so that it can travel through the crystal from one ion to the next, until it becomes trapped at a defect or sensitivity speck in the crystal, where it reacts with a silver ion to form an atom of silver. These atoms of metallic silver formed in a crystal are known as a latent

image. During subsequent development of the emulsion, the latent image catalyses the conversion of the entire crystal to silver. Any silver bromide crystals not reduced to silver are dissolved out of the emulsion by the fixative, resulting in a pattern of silver grains in the image of the light or radioactivity which hit the emulsion.

#### 3.1.6.2. Detection of *in situ* hybridisation.

*In situ* hybridisation can be detected with either X-ray film which gives an overall impression of hybridisation or with emulsions which allow more detailed resolution of hybridisation at the cellular level.

X-ray film can, for example, be used to localise hybridisation to particular areas of a tissue or embryo section. These areas can then be further analysed at the single cell level with nuclear emulsions. X-ray emulsions have large crystals with diameters ranging from 0.2 - 3.0  $\mu\text{m}$ , and are therefore more sensitive than nuclear emulsions which have smaller crystal sizes.

There are two types of emulsions, stripping film and nuclear emulsions. The use of stripping film involves the layering of a thin preformed film of emulsion over the cells. It gives adequate sensitivity and resolving power for analysis at the single cell level, and has the advantage of the reproducibility of a manufactured film for quantitative studies. However it does not give such close contact with the cells as nuclear emulsions.

The use of nuclear emulsion involves dipping the slide into a solution of molten emulsion. This results in very close contact between the cells and the emulsion, and by diluting the emulsion it is possible to form a very thin layer. Nuclear emulsion techniques therefore usually give superior results to stripping film. For high power magnification with the light microscope, nuclear emulsions give better resolution and are easier for staining and mounting under a coverslip due to the thinness of the film. The method of preparing the emulsion is quick and simple, and the emulsion

layer adheres to the slide and specimen much more firmly than stripping film. The major disadvantage of liquid emulsion relative to stripping film is that it is practically impossible to produce an emulsion layer of constant and reproducible thickness. This can cause problems when trying to quantitate hybridisation results, since it must be shown that variations in grain density are significant and not due to differences in the thickness of the emulsion. However, for isotopes of very low energy such as  $^3\text{H}$  and  $^{125}\text{I}$ , unevenness of the emulsion is less of a problem as long as the thickness of the emulsion is greater than the maximum path length of the particle.

#### 3.1.6.3. Sensitivity of nuclear emulsions.

Nuclear emulsions have a much higher concentration of silver bromide crystals than X-ray film and are available in a wide range of crystal sizes from 0.02 - 0.5 $\mu\text{m}$ . The smaller the crystal diameter the better the resolution achieved, but the harder it is to achieve a high sensitivity, since a small crystal can only contain a correspondingly small part of the path of a charged particle.

Nuclear emulsions are available in a range of sensitivities, so the most appropriate crystal size and sensitivity can be chosen for a particular application. The two main manufacturers of liquid emulsions are Eastman-Kodak (New York) and Ilford Ltd (Essex). Eastman-Kodak emulsions have a mean diameter of 0.2 $\mu\text{m}$  and are available in three sensitivities, NTB (for  $\alpha$  particles and low energy  $\beta$ -particles such as  $^3\text{H}$  and  $^{125}\text{I}$ ), NTB-2 (for  $\beta$ -particles up to 200keV energy) and NTB-3 (for highest energy  $\beta$ -particles).

Ilford offers the widest choice of products. Three crystal sizes are available, G (0.27 $\mu\text{m}$  mean diameter), K (0.20 $\mu\text{m}$ ) and L (0.15 $\mu\text{m}$ ). Each crystal is available in a range of sensitivities numbered from 0 - 5, with 5 being the most sensitive.



There are important differences between Ilford and Kodak emulsions, and techniques developed for one will not necessarily be suitable for use with the other. For example, Ilford emulsions are less sensitive to visible light, allowing brighter safety lighting, and do not require such vigorous drying procedures as Kodak during exposure, to prevent latent image fading.

#### 3.1.6.4. Problems with nuclear emulsions.

##### (a) Latent image fading.

This is caused by random recombination of silver and bromide atoms to reform silver bromide. The frequency with which this occurs increases with length of exposure, heat, the presence of water and the presence of oxidising agents. Therefore emulsions are exposed at 4°C in the presence of a drying agent such as silica gel. If latent image fading is still a problem, emulsions can be exposed in an O<sub>2</sub>-free atmosphere.

##### (b) Background.

The formation of background silver grains arises from a number of causes. The appropriate safety light filters must be used for the emulsion of choice to prevent exposure to light. Emulsions are most sensitive to light when in a dry state. Pressure can also cause the formation of a latent image. This may be due to either mechanical pressure, such as scratches or fingerprints, or stress caused by overdrying of the emulsion resulting from shrinkage of the gelatin when the emulsion is dried too rapidly. Ideally the emulsion should be allowed to gel before it starts to dry as this reduces stress. Some manufacturers, such as Ilford, add a plasticising agent to their emulsions (of which glycerol is an important component) to help reduce shrinkage of the gelatin during drying. During the processes of dipping slides and developing, this may be washed out of the emulsion, so it is often helpful to add 1% glycerol to all

solutions in contact with the emulsion. Stress can also be caused by the irregular surface of a section which may result in stress artifacts around the contours of the section.

Positive and negative chemography may be caused by chemicals present in tissues, resulting in the formation or loss of a latent image. However, fresh tissue is more likely to cause artifacts of this type than fixed and embedded tissue.

Since nuclear emulsions are highly sensitised, occasionally a crystal will spontaneously develop a latent image. The more sensitive the emulsion, the more likely it is to form a latent image in this way. Therefore emulsions should not be used that are more sensitive than required for a particular experiment. High temperatures cause an increase in the rate of spontaneous background formation. Therefore emulsions are stored and exposed at temperatures just above freezing.

#### 3.1.6.5. Histological techniques and autoradiography.

For *in situ* hybridisation, determination of the cellular structures underlying the silver grains is obviously as important as the detection of hybridisation itself. Therefore the histological methods used must be compatible with nuclear emulsion techniques. Most fixatives are compatible with emulsions. Coagulating fixatives have no effect on the emulsion. However, formaldehyde and glutaraldehyde can sometimes have a desensitising effect.

Sections embedded in paraffin wax must be completely dewaxed in fresh xylene before dipping in emulsion, since traces of wax on the slide will prevent even spreading of the emulsion and result in poor adhesion between the emulsion and the slide.

Some staining techniques need to be carried out before the emulsion is applied, for example where the reactive staining group will be destroyed or removed after exposure, development and fixation. Other techniques may cause heavy staining of the

gelatin or cause chemography resulting in blackening of the emulsion when used before exposure. As long as the stain does not react with groups present in the emulsion, post-exposure staining is generally the method of choice for most commonly used stains such as haematoxylin and eosin, and toluidine blue, since underlying cells can be easily stained through the emulsion.

### 3.2. MATERIALS AND METHODS.

#### 3.2.1. Tissue culture.

##### 3.2.1.1. Growth of Vero cells.

An African Green monkey kidney cell line (Vero) was used for all tissue culture experiments. These cells are susceptible to a wide variety of viruses that infect man. The cell line was maintained in Dulbecco's modification of Eagle's tissue culture medium supplemented with 10% newborn calf serum in 75cm<sup>2</sup> flask. The monolayer was split every 3-4 days when the cells were confluent by trypsinisation and seeding into 4-5 new 75cm<sup>2</sup> flask. Cells were incubated at 37°C in a CO<sub>2</sub>-gassed incubator.

##### 3.2.1.2. Sterilisation of slides.

Multiwell slides (C.A. Hendley (Essex) Ltd.) were cleaned by washing in absolute ethanol for 5 mins, then in dH<sub>2</sub>O 2-3 times and air-drying. Slides were sterilised by baking at 160°C for 2 h.

##### 3.2.1.3. Preparation of monolayers for *in situ* hybridisation.

Monolayers of Vero cells were grown on sterile multiwell slides. A confluent monolayer from a 75cm<sup>2</sup> flask was trypsinised and resuspended in 5-10mls medium. The cell concentration was determined using a haemocytometer and diluted to a concentration of  $2 \times 10^5$  cells/ml. 100 $\mu$ l ( $2 \times 10^4$  cells) were spotted onto each well of the slide in a sterile petri dish and incubated at 37°C. This concentration of cells was sufficient to give a confluent monolayer after overnight incubation. Monolayers of cells were infected as described below. Uninfected monolayers were used as negative controls.

#### 3.2.1.4. Infection of monolayers.

A working paramyxovirus stock was prepared (Appendix, Section VI). An appropriate virus dilution was prepared in medium supplemented with 2% calf serum and warmed to 37°C. The medium covering the monolayers was removed and 50µl of virus dilution was spotted onto each well of the slide. The virus was left to adsorb for 1 h at 37°C, then the slides were flooded with medium and incubated until a c.p.e. was seen. Slides were fixed as described below. Uninfected monolayers used as negative controls were flooded with medium and incubated as for virus-infected slides.

#### 3.2.2. **Fixation.**

A number of different fixatives were tested for use with *in situ* hybridisation. Fixed slides were used immediately or stored dessicated at -20°C until required, where they were stable for several months.

##### 3.2.2.1. Ethanol: acetic acid.

Cells were fixed in a solution of ethanol: acetic acid (3:1; v/v) for 15 mins at room temperature, then washed in absolute ethanol for 5 mins at room temperature and air dried. This method was routinely used for *in situ* hybridisation with DNA probes.

##### 3.2.2.2. Absolute ethanol.

Cells were fixed in absolute ethanol for 20 mins at 4°C and air dried.

##### 3.2.2.3. Methanol: acetone.

Cells were fixed in a solution of methanol: acetone (1:2; v/v) for 4 mins at -20°C and air dried.

#### 3.2.2.4. Acetone.

Cells were fixed in acetone for 4 mins at -20°C, then in acetone for 20 mins at 4°C and air dried.

#### 3.2.2.5. 1% formaldehyde.

Cells were fixed in a 1% formaldehyde solution in PBS'A' for 10 mins at room temperature, washed three times in PBS'A', dehydrated in graded alcohols and air dried.

#### 3.2.2.6. 5% formaldehyde, 2% sucrose.

Cells were fixed in a solution of 5% formaldehyde, 2% sucrose in PBS'A' for 10 mins at room temperature, washed three times in PBS'A', dehydrated in graded alcohols and air dried.

#### 3.2.2.7. 4% glutaraldehyde.

Cells were fixed in a solution of 4% glutaraldehyde in PBS'A' for 10 mins at room temperature, washed three times in PBS'A', dehydrated in graded alcohols and air dried.

### 3.2.3. *In situ* hybridisation with single-stranded DNA probes.

#### 3.2.3.1. Pretreatment.

Fixed slides which had been stored at -20°C were warmed to room temperature and permeabilised to increase penetration of the probe. Slides were incubated in 0.2M HCl for 20 mins at room temperature, washed briefly in dH<sub>2</sub>O, incubated in 10% formamide at 70°C for 30 mins (this step is optional and was generally omitted), washed briefly, and digested with 1µg/ml proteinase K in 20mM Tris-HCl pH7.4, 2mM CaCl<sub>2</sub> at 37°C for 15 mins. The cells were then washed twice for 5

mins each in dH<sub>2</sub>O, dehydrated in graded alcohols (70% for 5 mins twice, 95% for 5 mins once) and air dried.

### 3.2.3.2. Hybridisation to RNA.

The hybridisation solution was prepared as two separate components, solution A (20% dextran sulphate in freshly deionised formamide) and solution B (20mM Hepes buffer pH 7.2, 1.2M NaCl, 2mM EDTA, 2X Denhardts solution (0.04% Ficoll, 0.04% PVP, 0.04% BSA), 200µg/ml sonicated calf thymus DNA and 100µg/ml poly A). Both solutions were stored at -20°C.

For hybridisation, equal volumes of solution A and B were added to the required amount of probe and thoroughly mixed. The amount of probe used was <5µl per coverslip, in a final volume of 30µl per coverslip. Probes were used at a final concentration of 3-10ng/ml for M13 probes and 1-3ng/ml for oligonucleotides. Dithiothreitol (DTT) was added to a final concentration of 10mM to reduce the formation of disulphide bonds by the <sup>35</sup>S label. Hybridisation solution (30µl) was spotted onto the slide and a coverslip placed on top, covering four wells of the slide ensuring that there were no bubbles over the wells. The slides were placed in a light-proof box containing tissue saturated with hybridisation buffer to prevent the slides from drying out. Sealing of the coverslips was not necessary. The boxes were sealed with tape and hybridisation was carried out at 37°C for 24 h.

### 3.2.3.3. Post-hybridisation washing.

The slides were removed from the box and the coverslips gently floated off in a solution of 50% formamide, 0.6M NaCl, 80mM Tris-HCl pH 7.8, 4mM EDTA. The slides were washed twice at room temperature for 5 mins each in 2X SSC (20X = 3M NaCl, 0.3M sodium citrate), then for 1 h in 2X SSC at a high stringency temperature (60°C for M13 and oligonucleotide probes) and finally at room temperature for 2 h

in two changes of 2X SSC. The slides were then dehydrated in graded alcohols and air dried.

#### 3.2.3.4. Autoradiography.

Slides were either exposed to X-ray film for an overall analysis of hybridisation or coated with nuclear emulsion for analysis at the single cell level.

For exposure to X-ray film, dehydrated slides were covered with clingfilm and exposed at room temperature. Exposure time was generally 1-7 days for M13 probes and 1-3 weeks for oligonucleotide probes. The film was developed in Kodak D19 developer (diluted 1/5) for 5 mins at room temperature, washed in dH<sub>2</sub>O, fixed for 5 mins in Kodak fixative (diluted 1/5), and washed for 10-15 mins in running tap water before air drying.

For exposure to nuclear emulsion, slides were coated in the following way. A water-bath was set up in the darkroom at 42°C. Under Ilford 902 filter safety lighting, the required amount of Ilford K5 nuclear emulsion was measured out into a clean plastic tube, wrapped in aluminium foil and incubated in the water-bath for approximately 1 h to melt. A solution of 2% glycerol was also warmed to 42°C. Under safety lighting, the volume of emulsion was measured and an equal volume of glycerol solution added, mixed gently to avoid the formation of bubbles, and kept at 42°C until required. The diluted emulsion was poured into a petri dish standing on a heating block at 42°C to prevent the emulsion cooling. Slides were lowered section-side down into the emulsion and drained briefly. The slides were supported vertically in complete darkness for approximately 1 h to allow the emulsion to dry, then placed horizontally in light-proof boxes containing a sachet of silica gel and exposed at 4°C.

To develop the emulsion, the slides were warmed to room temperature, and developed in Kodak K19 developer (diluted 1/5) for 3 mins, stopped in 1% acetic



acid/1% glycerol for 1 min, and fixed in Kodak fixative (diluted 1/5) for 3 mins. The slides were gently washed in several changes of dH<sub>2</sub>O for at least 20 mins, then air dried. Cells were stained with either 1% toluidine blue or haematoxylin/eosin.

### **3.2.4. *In situ* hybridisation with single-stranded RNA probes.**

#### **3.2.4.1. Pretreatment.**

Cells were pretreated as for single-stranded DNA probes except that an acetylation step was added following proteinase K digestion, to reduce non-specific binding of probe. The slides were immersed in 0.1M triethanolamine pH8.0, acetic anhydride was added to 0.25% (v/v) and the solution mixed vigorously for 10 mins. Slides were washed and dehydrated as for use with DNA probes.

#### **3.2.4.2. Hybridisation to RNA.**

Hybridisation solutions were prepared as described for use with DNA probes with some alterations in components. Thus, solution A was composed of 20% dextran sulphate in formamide which had been purified by recrystallisation at least once. Solution B contained 20mM sodium phosphate pH6.8, 40mM Tris-HCl pH6.8, 1.2M NaCl, 5mM EDTA, 2X Denhardt's solution, 1mg/ml yeast RNA, 20mM DTT and 0.5% SDS. The hybridisation solution was prepared as described for DNA probes with the RNA probe being used at a concentration of 5-10ng/ml. Hybridisation was carried out at 50-55°C for 24 h.

#### **3.2.5.3. Post-hybridisation treatments.**

Following hybridisation, coverslips were removed as previously described, and the slides washed in 2X SSPE buffer (20X = 3.6M NaCl, 0.2M sodium phosphate pH7.0, 2mM EDTA), twice for 5 mins each at room temperature and once in 0.1X SSPE buffer at 40°C for 1 h. Non-specifically bound RNA was removed by digestion

with 50µg/ml RNase H in 2X SSPE at 37°C for 30 mins. Slides were then washed for 2 h in two changes of 2X SSPE at room temperature and dehydrated.

#### 3.2.5.4. Autoradiography.

Detection of hybridisation by autoradiography was carried out as described for DNA probes.

### 3.3. RESULTS.

#### 3.3.1. Detection of hybridisation.

Slides were initially exposed to X-ray film for general comparisons of relative hybridisation levels between infected monolayers. Hybridisation to groups of infected cells (syncytia or plaques) was indicated by spots on X-ray film, as shown for example in Figure 3.5. Slides were then coated with nuclear emulsion and examined at the cellular level. Hybridisation was seen only over plaques, as shown in Figure 3.2., confirming that hybridisation corresponded to morphological presence of virus.

The use of M13 and oligonucleotide probes labelled with  $^{35}\text{S}$  also gave sufficient resolution for detection of virus in single infected cells and allowed subcellular resolution of the hybridisation signal. For example, the cytoplasmic location of MV RNA in a single infected cell is shown in Figure 3.3.

#### 3.3.2. Optimisation of *in situ* hybridisation conditions for the use of single-stranded DNA probes.

There is a wide choice of methods recommended for *in situ* hybridisation. The method of Haase et al (1984a) was selected as a starting reference and adapted to give optimal results. Optimisation of conditions was carried out using cell monolayers infected with either MV or SV5. Virus stocks were generally used at multiplicities of infection (m.o.i.) that gave rise to discrete plaques, although SV5 was occasionally used at a m.o.i. that resulted in every cell being infected. Uninfected cells were used as negative controls for non-specific background binding.

##### 3.3.2.1. Hybridisation temperature.

Theoretical values of  $T_m - 25^\circ\text{C}$  for the M13 and oligonucleotide probes were in the range  $20-40^\circ\text{C}$ . Hybridisation temperatures of  $25^\circ\text{C}$ ,  $30^\circ\text{C}$  and  $37^\circ\text{C}$  were therefore tested for maximum hybridisation after a 24 h incubation time. The

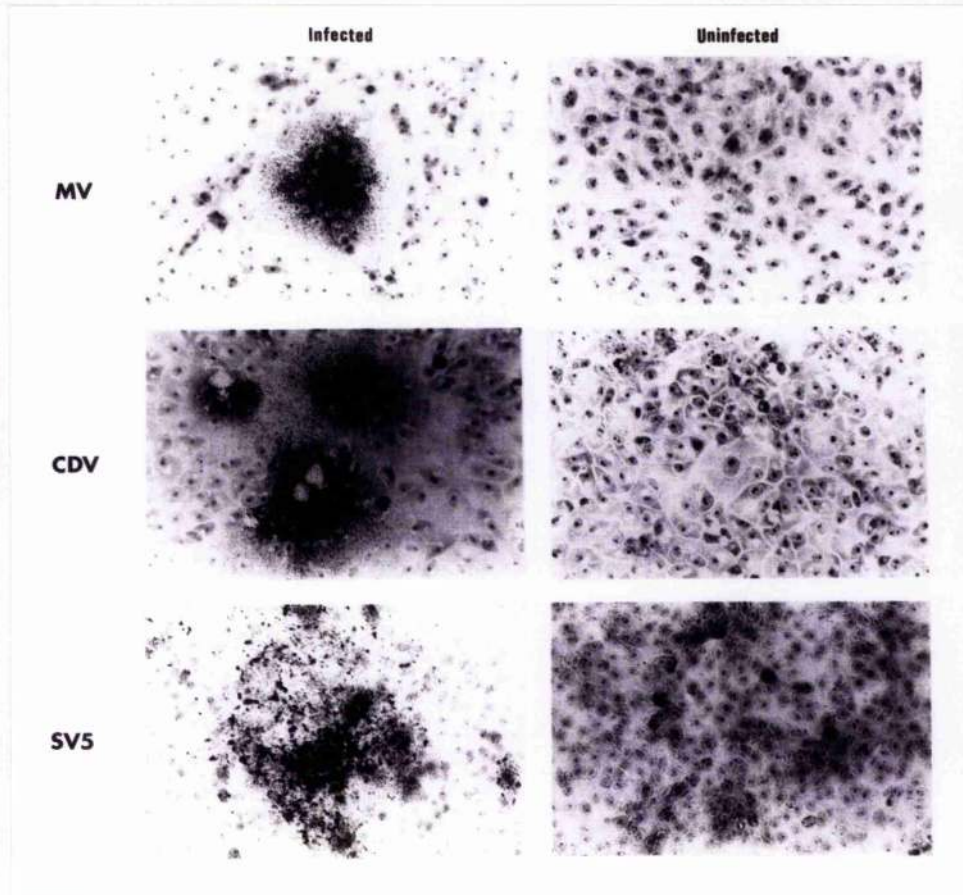
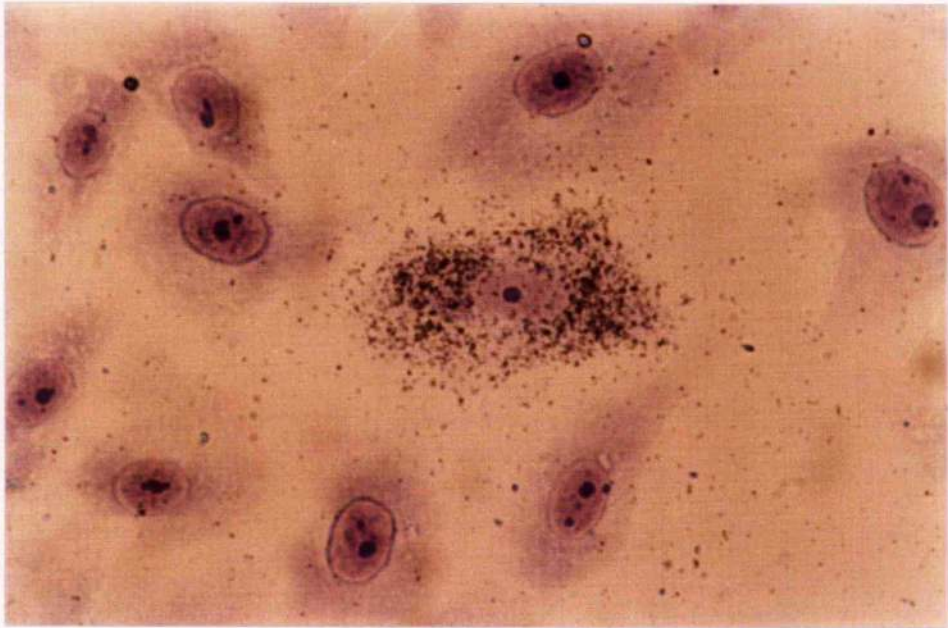


Figure 3.2. Detection of MV, SV5 and CDV in infected tissue culture cells by *in situ* hybridisation.

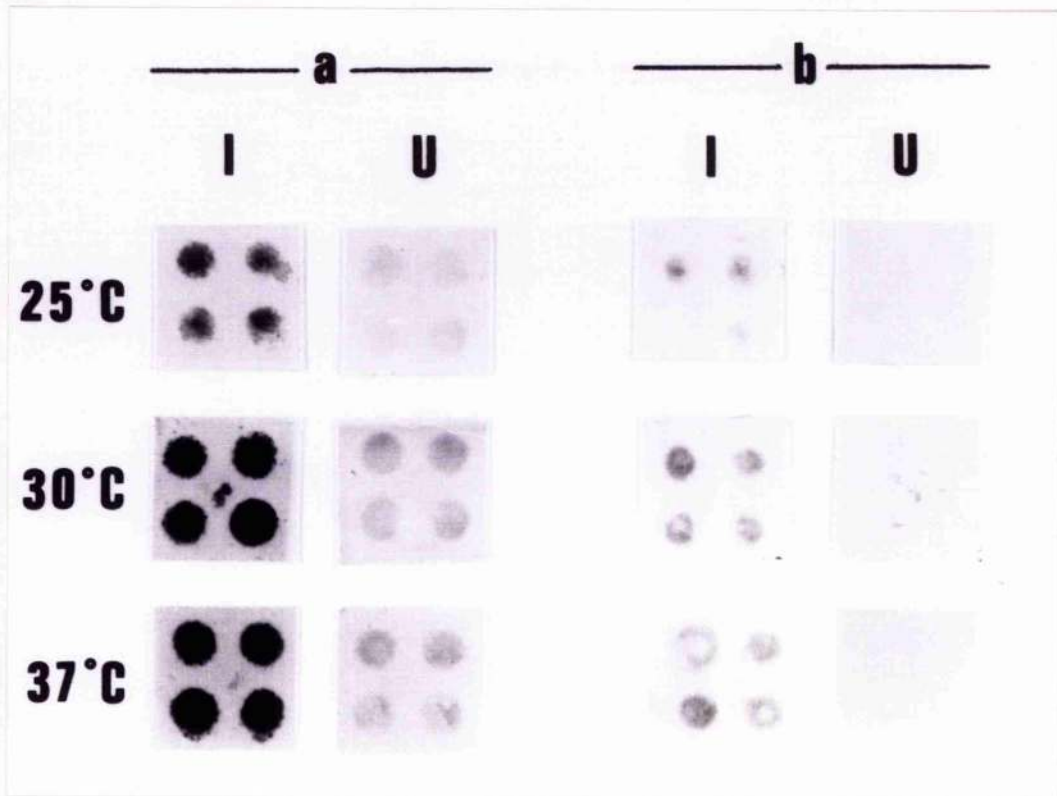
Monolayers of Vero cells were infected with MV, SV5 and CDV at a m.o.i. which gave rise to discrete plaques. Cells were fixed with ethanol:acetic acid 24-48 h p.i. when a c.p.e. was visible. Hybridisation was carried out using M13 probes which hybridised to MV NP gene (Z26), SV5 HN gene (4) and CDV NP mRNA (112H), described in Table 2.1. Slides were exposed to nuclear emulsion for 2 weeks. Stained with 1% toluidine blue. Magnification x150.





**Figure 3.3.** Detection of MV RNA in a single cell by *in situ* hybridisation.

Monolayers of Vero cells were infected with MV and fixed 24 h p.i. with ethanol:acetic acid. Cells were hybridised with an M13 probe (Z26) specific for MV viral RNA and exposed to nuclear emulsion for one week. The cytoplasmic location of the viral RNA is clearly visible.



**Figure 3.4. Determination of optimum hybridisation temperature for M13 and oligonucleotide probes.**

Monolayers of Vero cells were infected with a high m.o.i. of SV5 and fixed 24-48 hours p.i. with ethanol:acetic acid (3:1) when a c.p.e. was visible. Hybridisation was for 24 hours at the temperatures indicated. Probes used were (a) M13 probe 4 specific for the SV5 HN gene and (b) oligonucleotide probe **ABO35** specific for the SV5 HN mRNA. Slides were exposed to X-ray film for 2 weeks.

I = infected cells, U = uninfected cells.

temperature which gave maximum hybridisation signal was 30-37°C for both M13 and oligonucleotide probes (Figure 3.4.) but hybridisation signals, although less than maximal, could be detected over a wide range of temperatures (25-45°C, results not shown). A temperature of 37°C was used for all further experiments. Hybridisation at 25°C for 60-72 hours (Haase et al,1984a) gave a comparable signal (not shown) but was less convenient.

#### 3.3.2.2. Post-hybridisation washing stringency.

The maximum washing stringency was determined by increasing the temperature of the 1 hour post-hybridisation wash. The salt concentration was maintained at 0.33M (2XSSC) throughout. A temperature of 60°C was found to be the optimum for maximum stringency for both M13 and oligonucleotide probes (Figure 3.5.). Above 60°C a decrease in hybridisation signal was observed for both probe types.

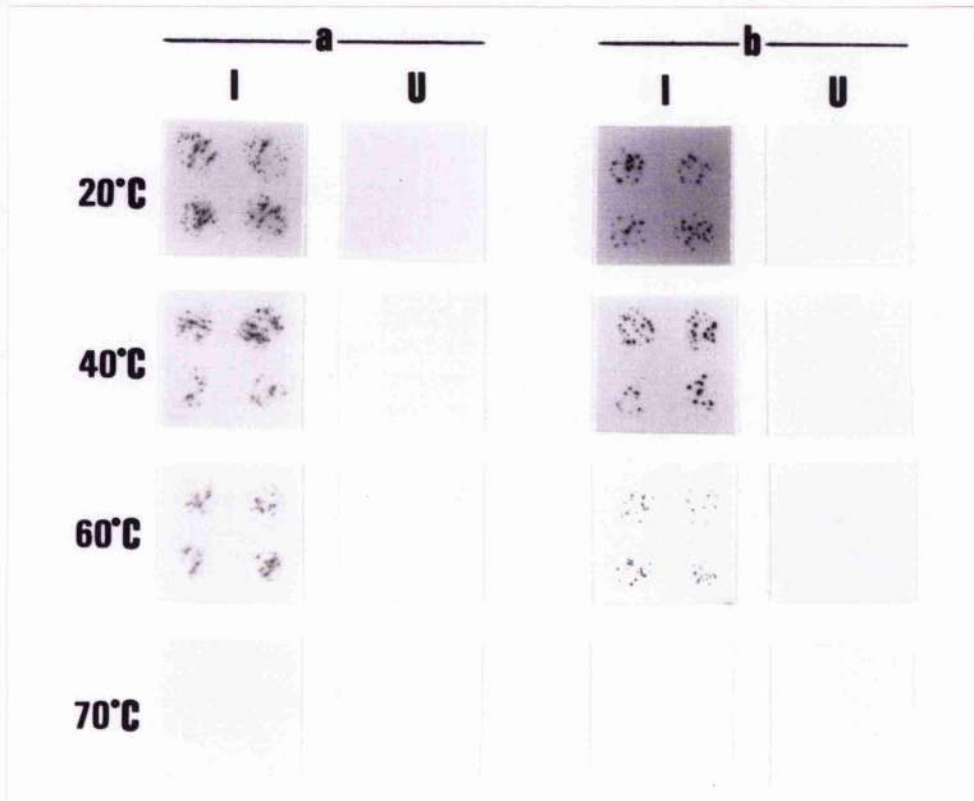
#### 3.3.2.3. Probe concentration.

For oligonucleotide probes, an increase in hybridisation signal was not observed above 1-3 ng/ml (Figure 3.6.(b)), therefore this concentration is in excess. Hybridisation could be detected using a concentration as low as 30 pg/ml. A higher concentration of M13 probe (10ng/ml) was required for maximal hybridisation (Figure 3.6.(a)) and the lowest concentration at which hybridisation could be detected was 0.3 ng/ml. In all following experiments, oligonucleotides probes were used at concentrations of 1-3 ng/ml and M13 probes at 3-10 ng/ml.

#### 3.3.2.4. Pretreatment steps.

The individual effect of the three pretreatment steps described by Haase et al (1984a) on hybridisation signal was investigated.

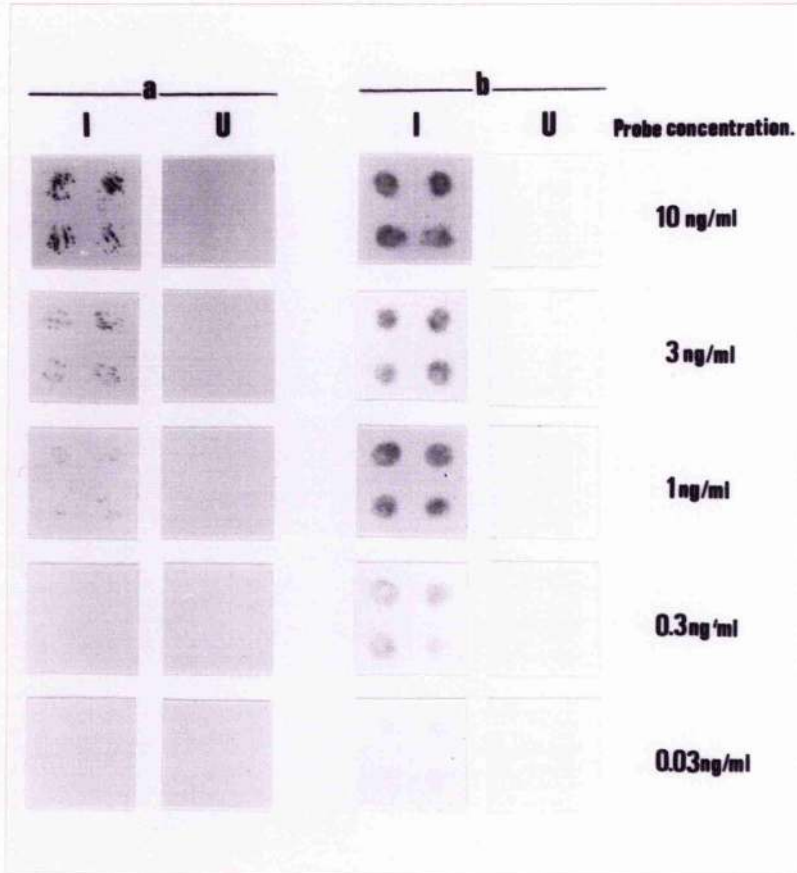




**Figure 3.5. Determination of optimum washing stringency for M13 and oligonucleotide probes.**

Monolayers of Vero cells were infected with either MV (a) or SV5 (b) and fixed 24-48 hours p.i. with ethanol:acetic acid (3:1) when a c.p.e. was visible. The high temperature post-hybridisation wash was carried out at the temperatures indicated. Probes used were (a) M13 probe 4 specific for the SV5 HN gene and (b) oligonucleotide probe 228924 specific for the MV N mRNA. Slides were exposed to X-ray film for 2 weeks.

I = infected cells, U = uninfected cells.



**Figure 3.6. Determination of optimum probe concentration for M13 and oligonucleotide probes.**

Monolayers of Vero cells were infected with SV5. Virus was used at either a high m.o.i. at which all cells were infected (**b**) or at a lower m.o.i. at which discrete plaques were formed (**a**). Cells were fixed 24-48 hours p.i. with ethanol:acetic acid (3:1) when a c.p.e. was visible. Probes used were (a) M13 probe 4 specific for the SV5 HN gene and (b) oligonucleotide **ABO35** specific for the SV5 HN mRNA and were used at the concentrations indicated. Slides were exposed to X-ray film for 2 weeks. I = infected cells, U = uninfected cells.

(a) High temperature incubation.

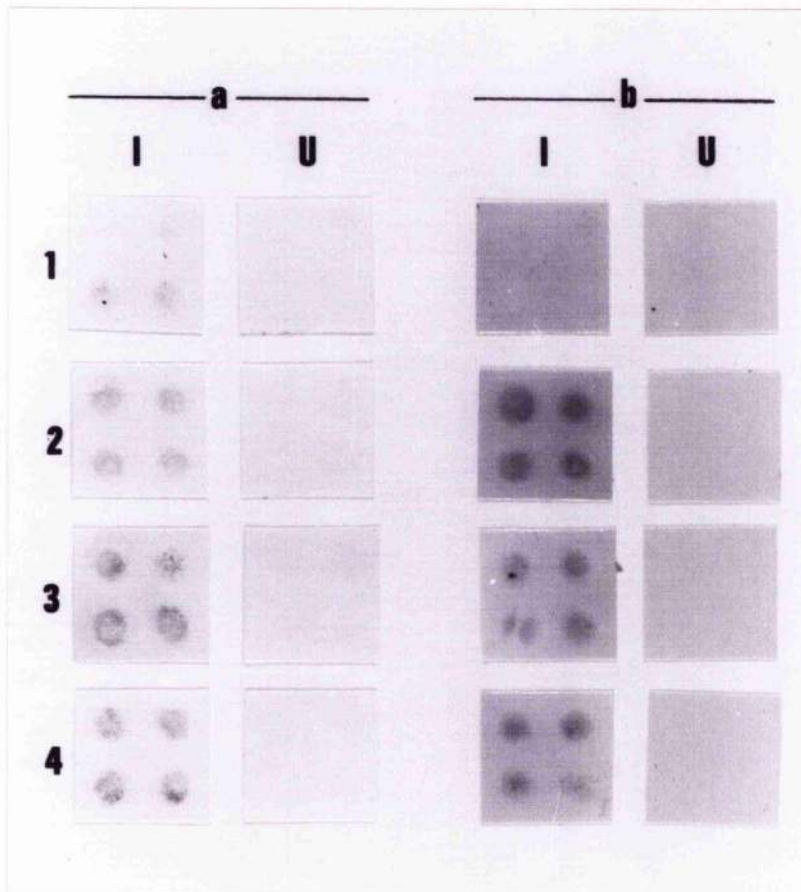
This step involved heating at 70°C for 30 minutes in 2X SSC (Haase et al,1984a). The effect of removal of salt and replacement with formamide solutions to destabilise RNA secondary structure was investigated. For both M13 and oligonucleotide probes, hybridisation signal was increased by removal of salt (Figure 3.7.). However the inclusion of formamide did not significantly increase hybridisation signal over incubation in water alone (Figure 3.7.). Omission of this step had little effect on hybridisation signal (see below) and sometimes caused loss of sections from the slide. Therefore this step was usually omitted.

(b) Effect of each pretreatment step on hybridisation signal.

The effect of each pretreatment step was further investigated using a number of different probes. The hybridisation signal obtained was compared for full pretreatment (i.e. acid, heat, proteinase K), each step alone and no pretreatment. The probes used were M13 4 which hybridises to SV5 HN gene (1.5 kb), M13 Z26 which hybridises to MV NP gene (1kb) and oligonucleotide 228924 which hybridises to MV NP mRNA (33 b).

For both the M13 probe Z26 and the oligonucleotide probe 228924, omission of pretreatment resulted in only a slight decrease in hybridisation signal (Figure 3.8.). In contrast, pretreatment was shown to have a significant effect on hybridisation with the SV5 probe 4. No pretreatment resulted in a dramatic decrease in hybridisation compared to that with full pretreatment. Investigation of the effect of each step alone showed that the effect of proteinase K digestion was to restore hybridisation signal to that obtained with full pretreatment, whilst acid and high temperatures alone had little effect.

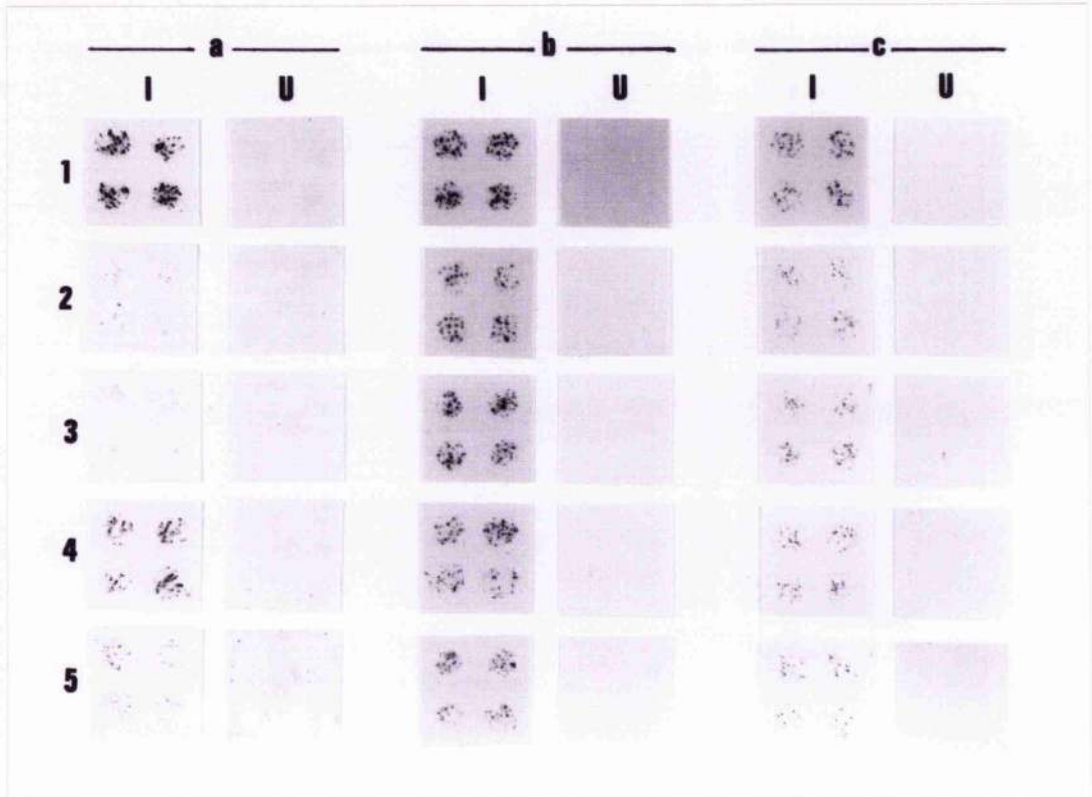




**Figure 3.7. Effect on hybridisation signal of variation of the conditions of the high temperature pretreatment step.**

Monolayers of Vero cells were infected with a high m.o.i. of SV5 and fixed 24-48 hours p.i. with ethanol:acetic acid (3:1) when a c.p.e was visible. The high temperature pretreatment step at 70°C for 30 minutes was carried out in the following solutions: (1) 2X SSC, (2) Water, (3) 10% formamide, (4) 50% formamide. Probes used were (a) M13 probe 4 specific to the SV5 HN gene and (b) oligonucleotide probe **ABO35** specific to the SV5 HN mRNA. Slides were exposed to X-ray film for (a) 1 day, (b) 1 week.

I = infected cells, U = uninfected cells.



**Figure 3.8. Effect of individual pretreatment steps on hybridisation signal using various probes.**

Monolayers of Vero cells were infected with either SV5 (a) or MV (b, c) and fixed 24-48 hours p.i. with ethanol:acetic acid (3:1) when a c.p.e was visible. Pretreatment was as follows: (1) Normal i.e. 0.2M HCl, 70°C in 10% formamide and proteinase K, (2) 0.2M HCl only, (3) 70°C in 10% formamide only, (4) proteinase K only, (5) No pretreatment. Probes used were (a) M13 probe 4 specific to the SV5 HN gene, (b) M13 probe Z26 specific to the MV N gene and (c) oligonucleotide probe 228924 specific to the MV N mRNA. Slides were exposed to X-ray film for 2 weeks.

I = infected cells, U = uninfected cells.

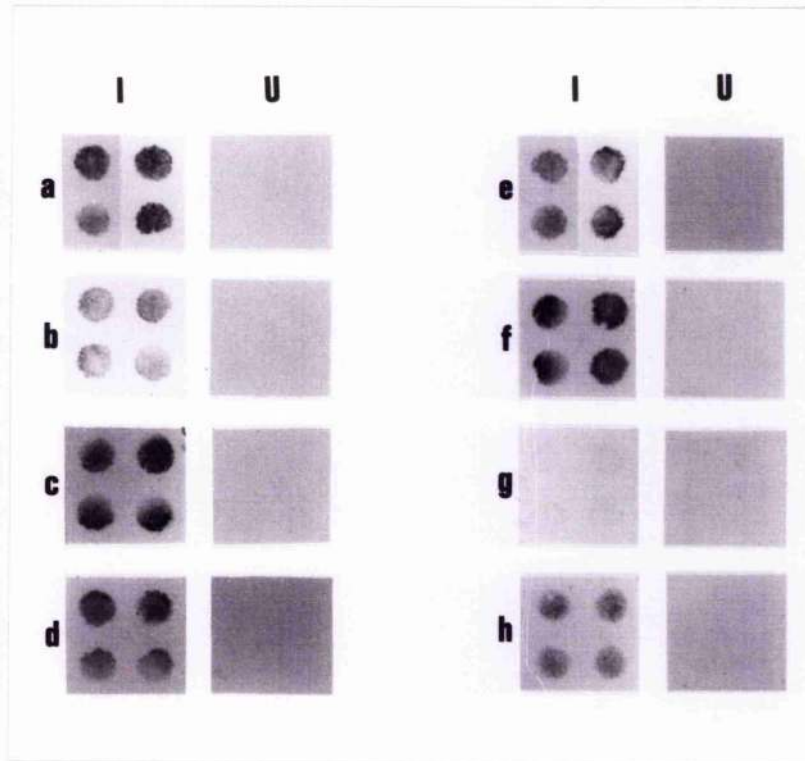
### 3.3.2.5. Fixation.

The effect on hybridisation signal of six fixatives commonly used for *in situ* hybridisation was compared- four coagulating fixatives (ethanol, ethanol:acetic acid, methanol and acetone) and two cross-linking fixatives (formaldehyde and glutaraldehyde). Slides were exposed to X-ray film for overall comparison of hybridisation levels. All fixatives allowed positive hybridisation to varying degrees when used with the M13 probe 4 (Figure 3.9.i.). Similar results were obtained using an SV5 oligonucleotide probe ABO35 (not shown). Of the coagulants tested, the weakest hybridisation signal was obtained with ethanol (Figure 3.9.i.b). However, the hybridisation signal obtained was improved by the use of ethanol in conjunction with acetic acid (Figure 3.9.i.a). Comparable hybridisation levels were obtained using acetone and methanol:acetone.

Positive hybridisation was seen in formaldehyde-fixed cells and an increase in hybridisation was obtained when the concentration of formaldehyde was increased from 1-5%. The fixative 5% formaldehyde, 2% sucrose is routinely used for immunocytochemistry, and therefore would be compatible for combined *in situ* hybridisation/immunocytochemical studies for the detection of nucleic acids and protein. In Figure 3.9.i.g, fixation was followed by permeabilisation with NP40, as used in immunocytochemical studies, rather than by deproteinisation as for *in situ* hybridisation as in Figure 3.9.i.h. As can be seen, NP40 treatment could not substitute for proteinase K treatment with this probe. Fixation with glutaraldehyde resulted in a reduction in hybridisation signal compared to formaldehyde.

Slides were coated with nuclear emulsion and examined at the cellular level. Relative comparisons were harder to make due to variations in the thickness of the emulsion. However, although there was some variation in the appearance of cells (Figure 3.10.), the cross-linking fixatives did not appear to result in a superior morphological preservation.





**Figure 3.9.i. Determination of the effect of various fixatives on hybridisation signal.**

Monolayers of Vero cells were infected with a high m.o.i. of SV5. Cells were fixed 24-48 hours p.i. when a c.p.e. was visible as follows. (a) Ethanol:acetic acid (3:1, v/v), (b) Absolute ethanol, (c) Methanol:acetone (1:2,v/v), (d) Absolute acetone, (e) 1% formaldehyde in PBS'A', (f) 5% formaldehyde, 2% sucrose (w/v) in PBS'A', (g) 5% formaldehyde, 2% sucrose (w/v) in PBS'A', (h) 4% glutaraldehyde in PBS'A'.

*In situ* hybridisation was carried out as in Materials and Methods with the exception of (g) where pretreatment was omitted and the cells were instead permeabilised with NP40 as for immunocytochemistry. The probe used was M13 4 specific to the SV5 HN gene. Slides were exposed to X-ray film for 5 days (i, above), then coated with Ilford K5 nuclear emulsion and exposed at 4°C for 2 weeks (ii, next page).

I = infected cells, U = uninfected cells.



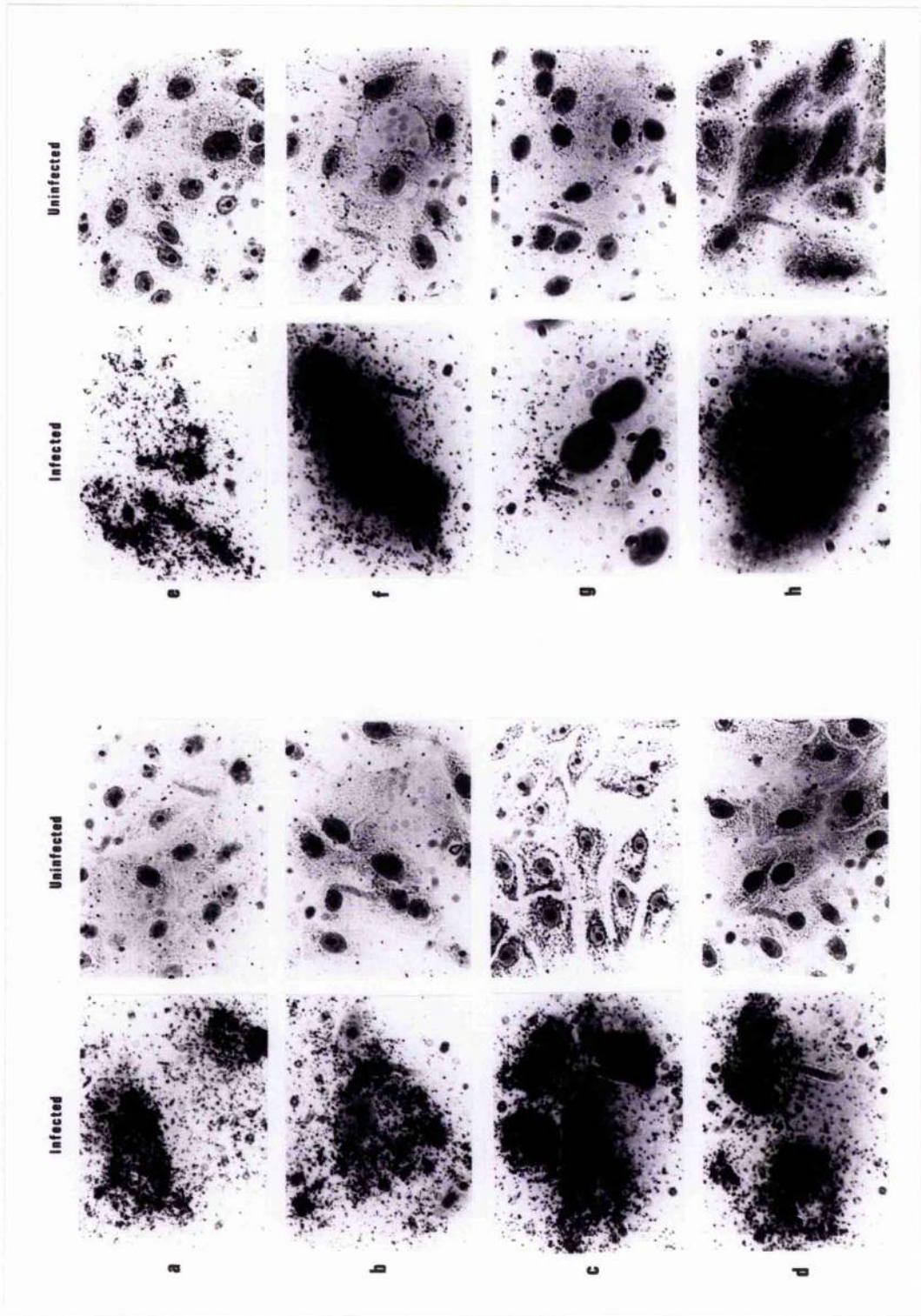


Figure 3.9. ii. (continued from previous page).

### 3.3.3. Optimisation of *in situ* hybridisation conditions for the use of RNA probes.

#### 3.3.3.1. Stability of RNA probes.

##### (a) Non-specific hybridisation to Vero cells.

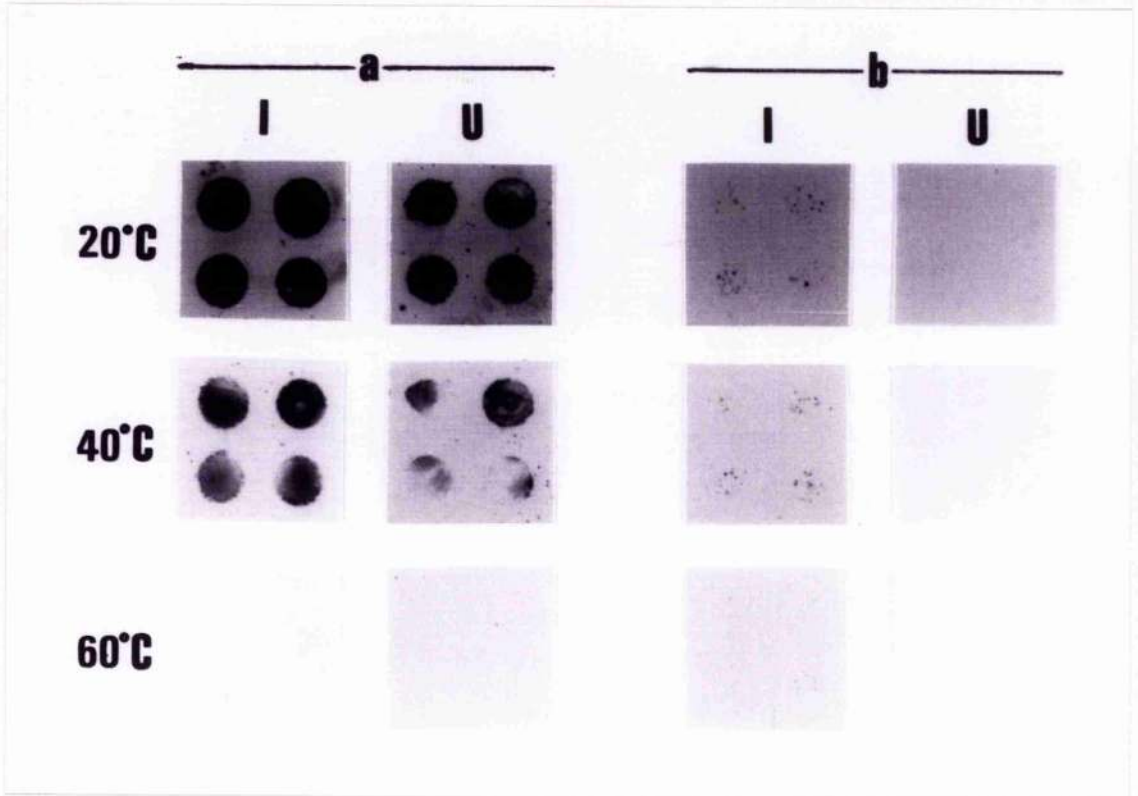
Riboprobes specific to MV (MVN and MVH) labelled as described in Chapter Two, were hybridised *in situ* to MV-infected vero cell monolayers. This was found to result in high levels of non-specific background hybridisation to the cell monolayer, compared to the specific hybridisation to individual plaques shown by the M13 probe Z26 (Figure 3.10.). This background binding was not removed by RNase digestion but was removed by increasing the post-hybridisation washing stringency using high temperatures in low salt concentrations (0.1X SSC) (Figure 3.10.) and was assumed to be caused by degradation of the RNA probe from RNase contamination, resulting in small fragments which could then bind to cellular RNA. The stability of the RNA probes during hybridisation was therefore further investigated and analysed by polyacrylamide gel electrophoresis.

##### (b) Stability in hybridisation solution.

The hybridisation solution used for DNA probes was initially also used for RNA probes. This is composed of two components, one solution containing dextran sulphate in deionised formamide and the other solution containing all other components (buffer, salt, carrier DNA, etc). The stability of RNA probes in each component was analysed by addition of equal volumes of probe and solution A or B, incubation overnight at 37°C, as for *in situ* hybridisation and electrophoresis on a 10% polyacrylamide/7M urea gel. Dextran sulphate was omitted from solution A to allow the sample to be run on the gel.

The stability of probe alone following labelling was shown by incubation and electrophoresis on a 10% polyacrylamide/7M urea gel. Addition of SDS to a final





**Figure 3.10. Non-specific hybridisation of RNA probes to Vero cells.**

Monolayers of Vero cells were infected with a m.o.i. of MV which resulted in discrete plaques and fixed 24 hours p.i. with ethanol:acetic acid (3:1) when a c.p.e was visible. Hybridisation was at 37°C for 24 hours. Post-hybridisation washes were in 0.1X SSC, and the high temperature wash was at the temperature indicated. Probes used were (a) RNA probe MVN specific for the MV NP mRNA labelled with  $^{35}\text{S}$ -UTP and (b) M13 probe Z26 specific for the MV NP gene labelled with  $^{35}\text{S}$ -dCTP. Slides were exposed to X-ray film for 3 days.

I = infected cells, U = uninfected cells.

concentration of 0.1% overcame the problem of RNase contamination during labelling as described earlier.

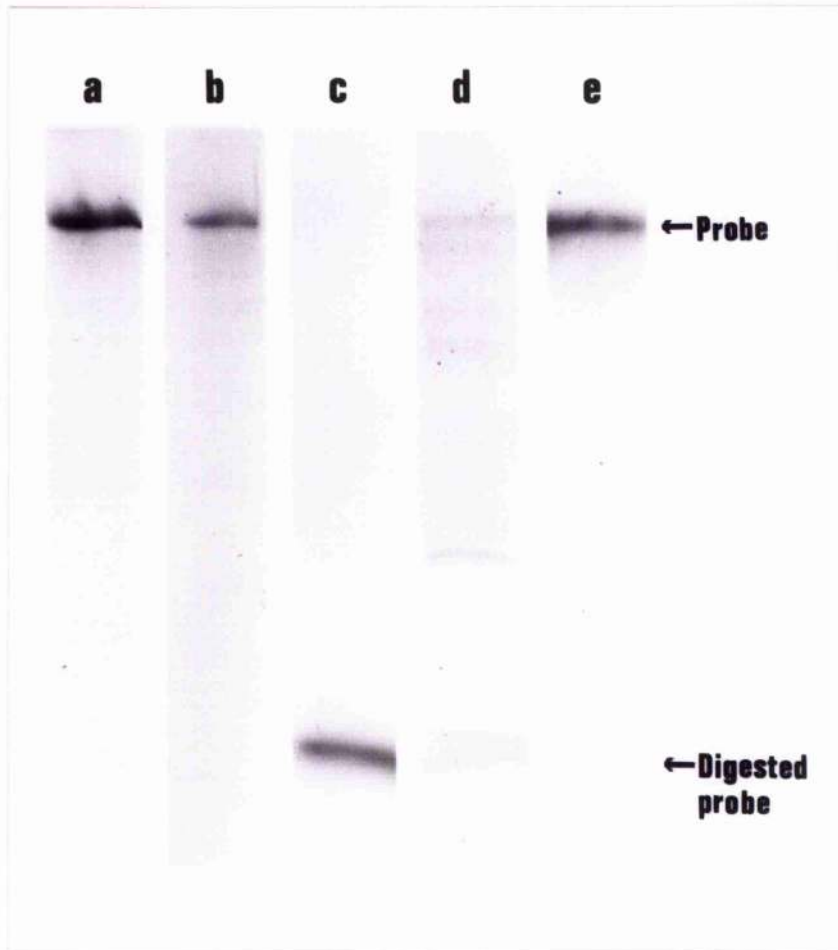
Incubation of probe with hybridisation buffer resulted in complete breakdown of probe (Figure 3.11. lane (c)). BSA was suspected to be the most likely contaminating agent but omission of BSA or replacement with RNase-free BSA did not prevent breakdown and therefore was not solely responsible. Degradation could be partially overcome by addition of SDS to 0.1% (Figure 3.11. lane (d)). However, stability was completely restored by addition of SDS to 0.5% (Figure 3.11. lane (e)).

Formamide is unstable and rapidly ionises to form ammonium formate, which results in an increase in pH up to pH9-10. Since RNA is degraded at high pH (above pH8), the stability of riboprobes in formamide alone was tested. Formamide was freshly deionised following manufacturers instructions i.e. stirred at room temperature for 30 minutes with a mixed-bed resin. Incubation of probe and deionised formamide resulted in partial degradation of the RNA (Figure 3.11. lane (b)). Buffering the formamide by addition of HEPES to 10mM (as used in the hybridisation solution) slightly decreased degradation but probe stability was completely restored by increasing buffer concentration to 50mM (Figure 3.12. lanes (c) and (d)).

A new hybridisation solution was prepared for the use of RNA probes, which in addition contained 0.5% SDS, a phosphate buffer, which has increased buffering capacity compared to HEPES, and which used formamide which had been purified by recrystallisation at least once. Incubation in this solution resulted in complete stability of RNA probes (Figure 3.13.).

#### 3.3.3.2. Fixation.

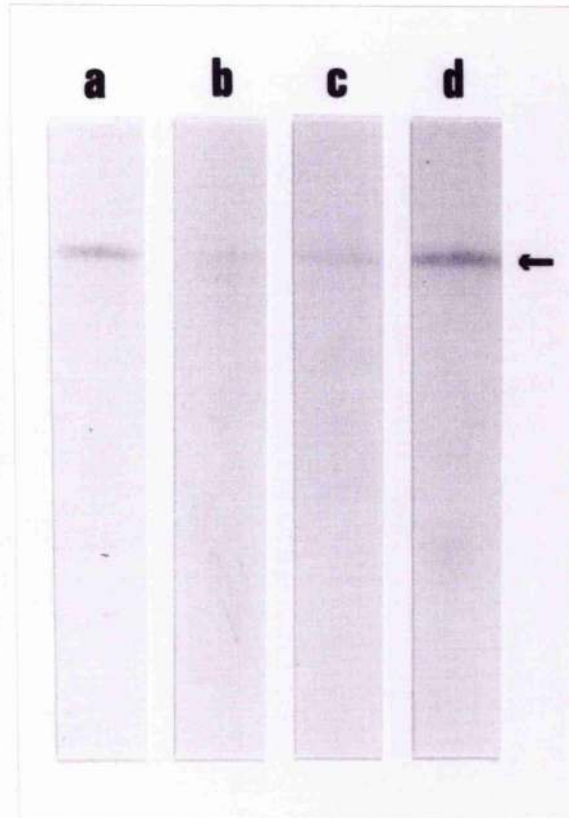
A coagulant fixative (ethanol:acetic acid) and a cross-linking fixative (5% formaldehyde) were tested for their effect on hybridisation with RNA probes. A



**Figure 3.11. Stability of RNA probes in DNA buffer solution.**

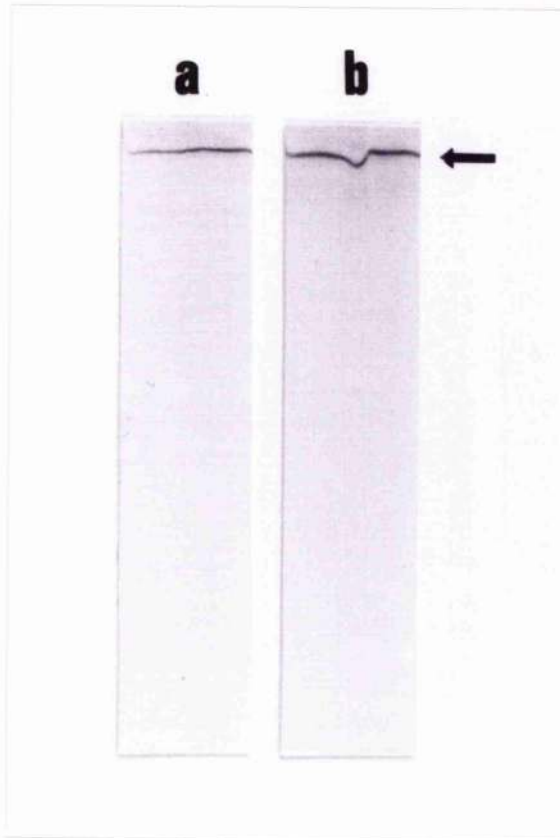
Hybridisation solution B contains all components of the final hybridisation solution except formamide and dextran sulphate. The stability of riboprobes during an overnight incubation at 37°C was tested by incubation of probe alone (track b) or equal volumes of probe and hybridisation solution B plus 0.1% SDS as below. Samples were analysed by denaturation in 50% deionised formamide (65°C, 5 mins) and electrophoresis on a 10% polyacrylamide /7M urea gel, followed by overnight exposure to X-ray film. (a) Probe stored at -20°C (not incubated), (b) Probe only, (c) Probe + hybridisation solution B, (d) Probe + hybridisation solution B + 0.1% SDS, (e) Probe + hybridisation solution B + 0.5% SDS.





**Figure 3.12. Stability of RNA probes in formamide.**

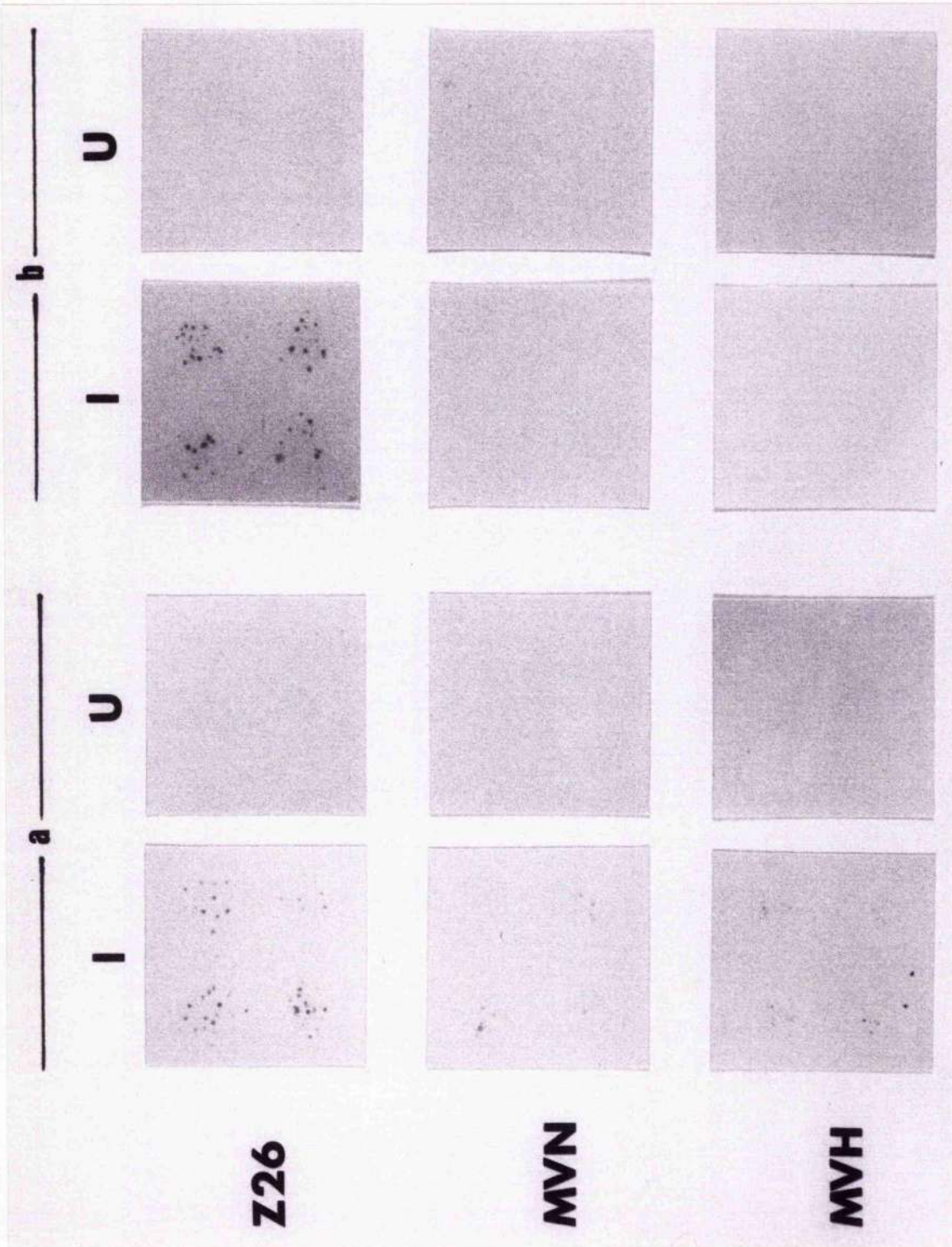
The stability of riboprobes in freshly deionised formamide was tested by overnight incubation at 37°C of equal volumes of probe, formamide and buffer as indicated below. Samples were analysed by electrophoresis on a 10% polyacrylamide/7M urea gel and overnight exposure to X-ray film. (a) Probe only, (b) Probe + formamide, (c) Probe + formamide + 10mM HEPES, (d) Probe + formamide + 50mM HEPES.



**Figure 3.13. Stability of RNA probes in RNA hybridisation buffer.**

A new hybridisation buffer was prepared for use with riboprobes (see text). Stability of riboprobes in this buffer was tested by overnight incubation at 37°C of equal volumes of probe and hybridisation buffer, followed by denaturation and electrophoresis on a 10% polyacrylamide/7M urea gel. The gel was exposed to X-ray film overnight. (a) Probe only, (b) Probe + hybridisation solution (all components except dextran sulphate).





**Figure 3.14. Hybridisation of RNA probes to MV-infected cells.**

Monolayers of Vero cells were infected with MV and fixed 24 h p.i. with (a) 5% formaldehyde, 2% sucrose or (b) ethanol:acetic acid

Probes used were the M13 probe Z26, specific for the MV N gene and the RNA probes MVN and MVH, specific for the MV N

and H mRNAs. Slides were exposed to X-ray film for 1 week.

I = infected cells, U = uninfected cells.

positive signal was obtained only with cells that had been fixed with formaldehyde (Figure 3.14.). Since a positive signal was obtained with both fixative types using an M13 DNA probe Z26, specific for MV (Figure 3.14.), this indicates a property of the RNA probes, rather than loss of RNA from the cells.

#### 3.3.3.3. Hybridisation temperature.

A range of temperatures were tested for optimal hybridisation (25-55°C) over a 24 hour hybridisation period (Results not shown). A positive hybridisation signal was obtained only at 50°C (Figure 3.14.) and at 55°C (not shown). The hybridisation signals obtained with both probes were very weak (Figure 3.14.).

### 3.4. DISCUSSION.

The method described by Haase et al (1984a) was chosen initially as a basis for these studies. This method had been shown to be applicable to the detection of viral RNA in tissue culture cells (Brahic & Haase, 1978; Haase, 1987) and for detection of persistent virus *in vivo* (Brahic et al, 1981). Each step of the method was examined and optimised for use with the three types of single-stranded probes described in Chapter Two. Hybridisation conditions were determined separately for the DNA (M13 and oligonucleotide) and RNA probes. All such experiments were carried out using an *in vitro* tissue culture system for detection of paramyxovirus RNA. This allowed reproducibility between samples enabling comparisons of relative hybridisation levels to be made. For most comparisons, the cells were exposed to X-ray film, rather than to nuclear emulsion for rapid analysis of results.

The use of M13 probes for *in situ* hybridisation gave the best results, with strong hybridisation signals after overnight exposure to X-ray film or with exposure to nuclear emulsion of up to one week. Although labelling of these probes was sometimes variable (see Chapter Two), their use in hybridisation studies was always consistent. The presence of low levels of agarose (<0.1%) from the labelling procedure in the hybridisation solution did not appear to affect hybridisation. The use of oligonucleotides for *in situ* hybridisation was also consistent although the hybridisation signal was generally weaker because of their smaller size.

Optimum temperatures for hybridisation and washing stringency determined experimentally were in the range indicated from theoretical calculations. The choice of oligonucleotide probe sequences with a high GC content (60-70%) resulted in hybrids of a strength comparable to the much larger M13 probes, as shown by a similar maximum post-hybridisation washing stringency. However, such a high GC content did not result in non-specific hybridisation to GC rich regions in uninfected cells, even with low stringency washes. The larger M13 probes tended to give higher levels of non-specific background hybridisation, presumably due to trapping of the

probe within the cells. The much smaller oligonucleotide probes were readily removed by washing and background hybridisation was never a problem.

Fixation is probably the most important step in *in situ* hybridisation. Adequate cellular morphology must be maintained for analysis of the hybridisation signal and cytoplasmic RNA must be fixed in position. However fixation must not be so extensive that the probe is unable to penetrate the cell. All initial experiments with DNA probes were carried out using cells fixed with ethanol:acetic acid (Haase et al,1984a). A comparison of this fixative with various other coagulating and cross-linking fixatives confirmed the suitability of this fixative for our system. Fixation with methanol:acetone or acetone alone was comparable to ethanol:acetic acid. However, the use of ethanol alone gave a decreased hybridisation signal, most probably due to loss of RNA from the cells. Interestingly, the positive hybridisation signal obtained with acetone contradicts the report by Haase (1987) that this fixative is incompatible with *in situ* hybridisation techniques. The use of formaldehyde also gave retention of RNA and a hybridisation signal comparable to ethanol:acetic acid. The decreased signal obtained with glutaraldehyde was most probably a result of inadequate probe penetration caused by "over fixation", since conditions had not been optimised for use with this fixative. Although differences in morphology of the cells with the various fixatives were apparent, the cross-linking fixatives did not appear to result in a superior morphology for studies at this level of magnification. Comparison of the results obtained by exposure to X-ray film and nuclear emulsion demonstrate the advantages of using X-ray film for rapid overall analysis of relative hybridisation levels.

There are widely conflicting reports of the most suitable fixative for *in situ* hybridisation, again reiterating the need for experimental determination in any particular system. Coagulating fixatives such as ethanol:acetic acid and methanol:acetone have been found to be superior for detection of viral RNA (Brahic & Haase,1978; Moar & Klein,1978; Haase et al,1984a; McCabe et al,1986) whereas



aldehyde fixatives are preferred by others for detection of mRNA (Singer & Ward,1982; Fournier et al,1983; Godard,1983; Lawrence & Singer,1985; McAllister & Rock,1985). The ability to use formalin-fixed paraffin-embedded tissues is particularly useful in allowing retrospective studies to be carried out (Blum et al,1984; Loning et al,1986; McDougall et al,1986; Ironside et al,1989).

The need for post-fixation treatment of cells to allow increased access of the probe varies depending on the fixative, cell type and probe used. Heating at 70°C in salt solution was found to reduce the hybridisation signal. This was assumed to be caused by loss of RNA from the cells caused by heating in the presence of salt as described by Pearce (1985), since heating in water or formamide solutions had no effect on hybridisation signal. This step was therefore usually omitted since it often caused loss of cells or sections from the slide. The combined effect of all pretreatment steps was a slight increase in hybridisation signal for the oligonucleotide 228924 and M13 Z26 probes, compared to the omission of pretreatment steps, although the individual steps did not show significant effects. Thus, incubation in dilute acid alone appeared to have little effect on hybridisation signal, although reported to enhance hybridisation by 2-3-fold (Gee & Roberts,1983). Digestion with proteinase K was found to have a significant effect on hybridisation signal only for the large M13 probe 4 (1.5kb). This effect appeared to be greater than previous reports of enhancement with proteinase digestion of 2-fold (Haase et al,1984a) and 5-fold (Gee & Roberts,1983), presumably due to the large size of this probe. The effect of proteinase K digestion on hybridisation signal obtained with the M13 Z26 probe (1kb) was much less significant, which could possibly be explained by differences between the two viruses, since it seems unlikely that the increase in size from 1 to 1.5kb could account for such a significant difference in probe penetration.

Initial experiments using RNA probes resulted in high levels of non-specific

background hybridisation to the cell monolayer. Since this was not removed by RNase digestion, but was reduced by increasing the stringency of washing, it was assumed that this effect was caused by degradation of the probe, resulting in small fragments of RNA which could bind to cellular RNA. Further investigation showed that these probes were unstable in the hybridisation solutions initially used for DNA probes. Thus degradation of probe occurred in the hybridisation buffer solution but could be prevented by the addition of SDS and was therefore presumably due to RNase contamination of one or more of the buffer components. Breakdown of probe also occurred when it was incubated with deionised formamide. Formamide is unstable and rapidly ionises to form ammonium formate, resulting in an increase in pH up to pH9-10. Since RNA is degraded at high pH, the formamide was further purified by recrystallisation at least once to remove ionic impurities. In addition, a phosphate buffer was included which has a stronger buffering capacity than HEPES and should therefore help prevent an increase in pH. These combined changes resulted in stability of the RNA probes.

Initial studies of the optimum hybridisation conditions required for RNA probes revealed a requirement for particular conditions. Thus hybridisation was detectable only in cells fixed with formaldehyde but not with ethanol:acetic acid, and only over a hybridisation temperature range of 50-55°C. Although most studies using RNA probes have used cross-linking fixatives (Angerer & Angerer, 1981; Cox et al, 1984), there are examples of their use with coagulant fixatives, in particular that of Cosby et al (1989) in which the same probes were used in sections fixed with ethanol:acetic acid and hybridised at 25°C. Further work needs to be carried out in order to explain these discrepancies.

The sensitivity of the RNA probes was very low and less sensitive than both the M13 and oligonucleotide probes. However, more detailed analysis of optimal conditions required for hybridisation were not possible due to time-constraints.

In conclusion, the use of RNA probes for *in situ* hybridisation was found to be

more problematic than the use of DNA probes, due to RNase contamination of solutions, the requirement for specific hybridisation conditions and a low sensitivity. In contrast, both M13 and oligonucleotide probes worked consistently and gave readily detectable hybridisation signals, although the preparation of these probes was more time-consuming. The use of DNA probes was therefore preferred in all subsequent studies for their stability, sensitivity and reproducibility.



## SECTION TWO.

In this section, the technique of *in situ* hybridisation has been applied to three areas of interest, using the optimal hybridisation conditions determined in Section One.

**CHAPTER FOUR.**

**Development of an animal model for studies  
of paramyxovirus-host interactions.**

## 4.1. INTRODUCTION

### 4.1.1 Use of animal models for studies of virus-host interactions.

The outcome of any virus infection is the result of complex interactions between the virus, the infected cells or cell types and the immune system of the host. Generally an effective immune response is mounted resulting in a short-lived self-limiting illness with complete eradication of virus. Less frequently, the destructive effect of virus replication results in serious illness or death. However, in some situations virus may not be completely eradicated, resulting in a persistent infection which may either be asymptomatic or may result in clinical symptoms becoming manifest months or years after the primary infection.

*In vivo* studies of virus-host interactions and the role of the host's immune system in eradication of virus are important for an understanding of the pathogenesis of infection and for the development of effective means of protection against viral infection through the administration of vaccines. The use of animal models of infection for such studies allows direct manipulation of the host and enables the effects on virus replication to be analysed within the context of the whole animal.

The development of techniques for direct detection of virus in infected tissue, such as immunofluorescence and *in situ* hybridisation, offer new opportunities for detailed studies of the pathogenesis of viral infections at the molecular level. In particular, *in situ* hybridisation offers the possibility for detection of virus in situations where restricted gene expression occurs, such as in persistent infections, where levels of viral proteins are often low or undetectable. The ability to detect specific gene transcripts by using single-stranded probes allows analysis of whether the infected cell supports full or only partial replication of the virus. Such techniques also enable identification of specific cell types involved in supporting replication and spread of the virus together with direct analysis of the morphological changes undergone in affected tissues.

In this chapter, we demonstrate the use of *in situ* hybridisation for studies of the replication of the paramyxovirus, simian virus 5 (SV5), in a mouse model system, with particular emphasis on the mechanisms of clearance of virus from infected tissue and the effects of immunisation on the speed of virus clearance. Mice were anaesthetised and infected intranasally with SV5. At various times post-infection, mice were sacrificed and the level of virus replication in the lungs was assessed by hybridisation to the viral RNA using a single-stranded M13 DNA probe. Levels of virus proteins were also determined by immunofluorescence. Detection of virus *in situ* also allowed morphological changes in the infected tissue to be analysed in relation to the presence of virus.

This system allowed changes in viral replication, brought about by perturbation of the host's immune system, to be compared to levels of replication and clearance of virus in normal mice. This model was thus used to investigate two areas of interest, firstly as a system for assessing the protective effect of viral vaccines and secondly as a possible model of paramyxovirus persistence.

#### **4.1.2. Introduction to paramyxoviruses.**

SV5 is a member of the paramyxoviruses. This genus includes significant human pathogens, such as measles virus and respiratory syncytial virus, which are major causes of respiratory illness and death in infants, particularly in developing countries (Assaad, 1983; Stott & Taylor, 1984). A model of SV5 infection is therefore relevant to a general understanding of the immune response involved in protection against such viruses and in the development of effective vaccines.

Three distinct subgroups of paramyxoviruses have been defined, the parainfluenzaviruses, the morbilliviruses and the pneumoviruses (Kingsbury et al, 1978). The parainfluenzaviruses are ubiquitous viruses which infect man, mammals and birds, and include mumps virus, Sendai virus of mouse, the human parainfluenzaviruses (PF 1-4) and SV5. The morbilliviruses include the closely

related measles virus (MV) and canine distemper virus (CDV) which are associated with exanthematous, and occasionally neurological disease, in addition to respiratory disease. The pneumovirus subgroup, which includes respiratory syncytial virus (RSV), is more distantly related and may eventually be grouped separately.

The paramyxoviruses are the largest of the RNA viruses and have a pleomorphic structure composed of an internal ribonucleoprotein core or nucleocapsid, surrounded by a host cell-derived lipid envelope (Figure 4.1.). The nucleocapsid contains the single-stranded negative sense genomic RNA, complexed with nucleoprotein (NP). Also found as an integral part of the nucleocapsid are the large (L) and polymerase (P) proteins, which together form the virus-specific RNA polymerase. This complex can carry out all transcriptional activities including polyadenylation, capping and methylation. The lipid envelope contains two transmembraneous glycoproteins, the haemagglutinin-neuraminidase (HN) and the fusion protein (F). As its name suggests, the HN protein has both haemagglutinating activity and neuraminidase activity (Scheid et al,1972). The haemagglutinating activity is responsible for adsorption of the virion to the cell surface via neuraminic acid-containing receptors. The function of the neuraminidase activity is not known but is thought to be involved in aiding release of the virus from the cell surface by removing neuraminic acid from cell surface receptors. The morbilliviruses have no neuraminidase activity and the protein is designated H. The pneumoviruses have neither activity and the protein is designated G. The nature of their cellular receptors is unknown. The fusion protein allows penetration of the virion to the cell by fusion of virus and host membranes (Scheid & Choppin,1974; Choppin & Scheid,1980). The structure of the envelope and the overall integrity of the virion is maintained by the matrix (M) protein which is located beneath the lipid envelope and forms a link between the nucleocapsid and the envelope (Tyrrell et al,1980). A number of other structural and non-structural proteins have

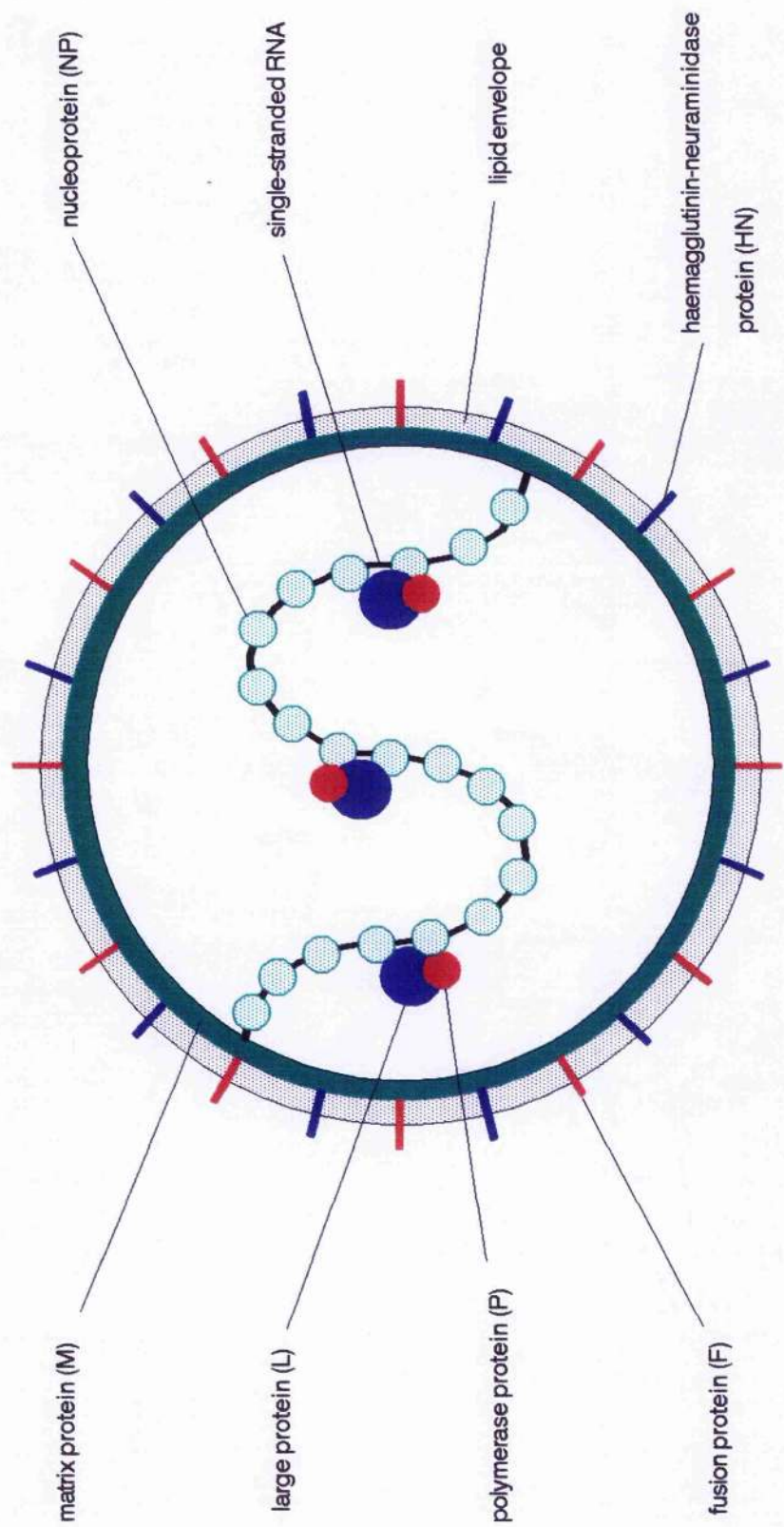


Figure 4.1. Diagram of a paramyxovirus.



recently been identified, although their functions are as yet unknown.

Replication of paramyxoviruses begins with attachment of the virion to cellular receptors, rapidly followed by fusion of the viral and cell membranes. Fusion results in release of the nucleocapsid into the cytoplasm. Paramyxoviruses replicate in the cytoplasm, and primary transcription occurs independent of host cell protein synthesis by the viral RNA polymerase (L and P proteins). Translation of primary mRNAs results in the synthesis of viral proteins and amplified transcription follows. With the production of viral proteins, RNA replication begins, resulting in the synthesis of full-length positive-sense RNA which then serves as a template for the synthesis of negative-sense genomic RNA. The two mutually exclusive functions of transcription and replication are both carried out by the viral RNA polymerase and the choice between these two functions is determined by the levels of nucleoprotein in the cell, which binds to an initiation site of nucleocapsid assembly in the leader sequence.

Transcription starts from a single promoter at the 3' end of the genome (Collono & Banerjee, 1977; Emerson, 1982) and sequential transcription occurs in the order 3'-NP-P-M-F-HN-L-5' (Dowling et al, 1986; Rima et al, 1986; Shioda et al, 1986; Spriggs et al, 1986), with initiation and termination occurring between each gene. This results in a gradient of mRNAs decreasing from NP to L, as the probability of the polymerase remaining attached to the genomic RNA decreases (Abraham & Banerjee, 1976; Ball & White, 1976).

Translation of mRNAs occurs on host cell ribosomes and assembly takes place at the plasma membrane of the cell. The viral lipid envelope is derived entirely from host cell membrane (Patzner et al, 1979) and is modified during assembly and budding resulting in the total exclusion of host cell proteins. Nucleocapsids are assembled in the cytoplasm and diffuse to the cytoplasmic face of the plasma membrane. The matrix protein appears to associate rapidly with the plasma membrane and cross-links the viral glycoproteins and nucleocapsid (Wilson &

Lenard,1981; Emerson,1988). Binding of nucleocapsid is rapidly followed by bud formation, in which the modified membrane bulges out and is nipped off releasing the virion.

A frequent feature of paramyxovirus infections is the formation of multinuclear cells or syncytia. This happens when the F protein precursor ( $F_0$ ) is cleaved, and hence activated, allowing fusion to occur with contiguous membranes. This allows the rapid spread of virus from cell to cell (Gallaher & Bratt,1974).

#### **4.1.3. Immune response to paramyxoviruses.**

The normal host immune response to viral infection consists of complex interactions between the humoral and cell-mediated arms of the immune response. Infection results in activation of B cells which divide into memory B cells and differentiated or plasma cells which secrete different clones of antibodies. B cell responses are usually T-helper cell dependent. Production of antibodies that interact with the HN or the F protein may result in inactivation or neutralisation of cell-free virus.

The cell-mediated immune response results in the production of cytotoxic T cells which can kill virus-infected cells, thus preventing further spread of virus. Soluble lymphokines, such as  $\gamma$ -interferon, may also be released by T cells. These result in the accumulation of activated macrophages at the site of infection and may also interfere directly with virus replication. The cell-mediated response is initiated by the presentation of antigen to T lymphocytes by antigen-presenting cells. In contrast to B cell recognition, T cells may recognise target antigens on either internal or external viral proteins. This is because T cells interact with degraded forms of antigen which have been internally processed by the cell and presented at the cell surface in conjunction with molecules of the major histocompatibility complex (MHC). T cell recognition is thus said to be

MHC-restricted. Circulating T cells are classified into two main populations defined by cell surface antigens, known as CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Reinherz et al,1979). Although there is some functional overlap between these two subpopulations, they can be distinguished by the way in which they recognise antigen. Thus, CD8<sup>+</sup> cells recognise antigen only in conjunction with Class I MHC molecules (Class I-restricted) whereas CD4<sup>+</sup> cells are Class II-restricted. Class I molecules are found on the surface of almost all nucleated cells. Class II antigens are found on antigen-presenting cells which are involved in initiating the immune response, such as B cells, activated T-cells and macrophages. For most viral infections, cytotoxic T cells are Class I-restricted, although for some viruses, such as measles, Class II-restricted cytotoxic lymphocytes may also be important.

The relative importance of the humoral and cell-mediated arms of the immune response in protection and recovery from most viral infections is still far from clear. During infection with a number of paramyxoviruses and the closely related orthomyxoviruses, the production of neutralising antibodies against the surface glycoproteins has been shown to be protective *in vivo* (Schulman et al,1968; Virelizier et al,1976; Taylor et al,1984; Giraudon & Wild,1985; Wolinsky et al,1985; Love et al,1986). However, the cell-mediated immune response also appears to play an important role in clearance of such infections, as demonstrated in a number of animal models. Thus, during infection of mice with influenza virus, an orthomyxovirus, cytotoxic T cells have been shown to mediate protection and recovery (Taylor & Askonas,1986; Wraith et al,1987; Mackenzie et al,1989). Studies of RSV infection have demonstrated the development of a cytotoxic response in mice infected intranasally with live virus (Kumagi et al,1985; Taylor et al,1985) and the presence of memory T cells was demonstrated in the spleen of such mice (Bangham et al,1985). It has also been shown that following infection of immunodeficient mice with RSV, virus can be cleared by transfer of RSV-primed T cells, indicating their importance in recovery from infection (Cannon et al,1987).

Similarly, work in our laboratory has shown that virus-specific cytotoxic T cells can be demonstrated in mice infected with SV5 (Randall et al,1988; Randall & Young,1988). The development of a cytotoxic T cell response therefore seems to play an essential role in protection against these viruses.

#### **4.1.4. Development of subunit paramyxovirus vaccines.**

The general approach to the production of virus vaccines has been in the use of attenuated or whole killed virus. However, for many infections, there may be inherent difficulties in the use of such vaccines. This is illustrated by RSV infection, in which prior administration of a killed virus vaccine results in a more severe illness following subsequent infection (Kim et al,1969; Fulginiti et al,1969). Therefore much recent research has concentrated on developing subunit vaccines in which a restricted number of virus antigens are used to induce immunity.

The production of subunit vaccines has so far mainly concentrated on the incorporation of hydrophobic proteins into micelles (Morein et al,1984; de Vries et al,1988; Morein,1988), the use of infectious expression vectors such as vaccinia (Drillien et al,1988; Moss et al,1988), or the use of synthetic peptides, either attached to adjuvants or incorporated into virus chimaeras (Bittle et al,1982; Cohen et al,1984; Evans et al,1989). However, recent work in our laboratory has investigated the use of purified viral proteins as immunogens and has involved the development of a new type of subunit vaccine, described as solid matrix-antibody-antigen (SMAA) complexes which have been used to induce protective immunity in mice by immunisation with SV5 proteins (Randall et al,1988; Randall & Young,1988). The basis of this type of vaccine is the attachment of virus-specific monoclonal antibodies to a solid matrix, which is then used to purify the viral protein against which the antibody has been raised. The whole complex is then used for immunisation. This method of vaccine production

has the advantage of a rapid, simple, non-denaturing protein purification step, and also has the potential to simultaneously immunise against more than one protein from more than one virus (Randall,1989; Randall & Young,1989).

It would be useful to have a convenient model system for assessing the effectiveness of different vaccines in protection against infection, and for determining the specific immune mechanisms involved in such protection. We have therefore been developing a mouse model system for SV5 to see if it could be applied to such studies. Here, the protection induced by immunisation with various purified SV5 proteins, presented as SMAA complexes, was assessed by comparison of the levels of viral RNA in the lungs of immunised and non-immunised infected mice. A comparison of levels of viral protein was also assessed by direct immunofluorescence in the lungs.

#### **4.1.5. Animal models of paramyxovirus persistence.**

It has long been recognised that paramyxoviruses have the ability to persist *in vitro*, and it is now evident that persistence *in vivo* also occurs, often resulting in chronic disease symptoms. Although the exact mechanisms of persistence have yet to be established, a number of likely mechanisms have been suggested by studies *in vitro* and *in vivo*. These involve both defects in the normal productive replication cycle, resulting in the absence of infectious virus particles, and factors involving an abnormal immune response. These mechanisms and the association of paramyxoviruses with a number of chronic human diseases are discussed in more detail in Chapter Five.

The role played by the host's immune system is likely to be of paramount importance in permitting the establishment of persistent infections. This is illustrated by subacute sclerosing panencephalitis, a chronic human disease caused by a persistent measles virus infection in which persistence of virus occurs despite an apparently normal recovery from acute infection, and the presence of

high levels of neutralising antibodies in the cerebrospinal fluid (CSF) and serum. The ability to establish a persistent paramyxovirus infection in an animal model would allow more detailed studies of the host and viral factors involved in such infections.

There are few animal models of paramyxovirus persistence. However, it has previously been shown that a persistent infection of RSV can be induced in immunodeficient mice (Cannon et al, 1987). The ability of SV5 to establish a persistent infection in mice made immunodeficient by X-irradiation has therefore been investigated.



## 4.2. MATERIALS AND METHODS.

### 4.2.1. Infection of mice.

Mice were anaesthetised with ether and infected intranasally by inhalation of  $5 \times 10^6$  -  $10 \times 10^6$  p.f.u. of the human isolate (LN) strain of SV5 (Goswami et al, 1984) in a volume of 80  $\mu$ l of growth medium. At various times post-infection, pairs of mice were sacrificed and the lungs removed. Collapsed lungs were removed and frozen directly in liquid nitrogen. Inflated lungs were prepared by injection of 1-2 mls of Bright embedding fluid (diluted 1:1 with PBS'A') into the trachea of partially removed lungs until they were fully inflated. The lungs were then fully removed and frozen in liquid nitrogen. Frozen lungs were stored at  $-70^{\circ}\text{C}$  until required. Inflation of the lungs prior to freezing allowed complete maintenance of the normal physical structure of the lung, with alveoli clearly visible.

### 4.2.2. Preparation of lung sections.

Sections of 10  $\mu$ m thickness were cut using a Bright OTF/AS cryostat and mounted on activated organosilanated slides (see Appendix, Section V). Sections were air dried briefly and fixed with ethanol:acetic acid (3:1) for *in situ* hybridisation (Section 3.2.3.) or with 5% formaldehyde, 2% sucrose for immunofluorescence (Section 4.2.4.).

### 4.2.3. *In situ* hybridisation for detection of viral RNA.

*In situ* hybridisation was carried out as previously described (Section 3.2.4.). Lung sections were pretreated with 0.2M HCl, digested with 1  $\mu$ g/ml proteinase K and dehydrated in graded alcohols. The high temperature incubation step was omitted to reduce loss of sections from the slides. Hybridisation was carried out at  $37^{\circ}\text{C}$  for 24 hours using the M13 probe 4, specific for the SV5 viral HN gene, at a concentration of approximately 3ng/ml. Post-hybridisation washes included a high stringency wash at  $60^{\circ}\text{C}$  in 2X SSC. Slides were coated with Ilford K5 nuclear emulsion and

exposed at 4°C for 1-2 weeks. Following development, slides were stained with haematoxylin and eosin.

#### **4.2.4. Direct Immunofluorescent detection of virus protein.**

Lung sections were fixed with 5% formaldehyde, 2% sucrose in PBS'A' for 10 mins at room temperature and washed again. The cells were permeabilised by treatment with 0.5% NP40, 10% sucrose in PBS'A' for 5 mins and washed three times in PBS'A'. The presence of virus was detected using a mixture of monoclonal antibodies raised against the SV5 P protein. The slide was dried around the section and a volume of FITC-labelled antibody (diluted to approximately 0.1mg/ml in PBS'A') was applied to cover the section. Slides were incubated for 1 h in a dark humidified box. Slides were then washed once with permeabilisation buffer and 4-5 times with PBS'A'. Sections were mounted in 50% glycerol under a coverslip and viewed under a fluorescent microscope.

#### **4.2.5. Indirect Immunofluorescent detection of mouse macrophages and T-cells.**

Fixed and permeabilised lung sections were tested for the presence of mouse macrophages (mo), cytotoxic T-cells (CD8<sup>+</sup>) and helper T-cells (CD4<sup>+</sup>) using rat monoclonal antibodies specific for each cell type. Binding of antibody was detected using a goat anti-rat polyclonal antibody, labelled with FITC and adsorbed as described above.

#### **4.2.6. Immunisation of mice with solid matrix-antibody-antigen complexes.**

Solid matrix-antibody-antigen complexes were prepared by R.E. Randall and D.F. Young as follows. Staphylococcus aureus (Staph.A)-antibody complexes were prepared by incubating equal volumes of ascitic fluids with 'fixed' and killed

suspensions of the Cowan A strain of Staph.A (10% w/v; Randall & Young,1988). Unbound antibody was removed by centrifugation and resuspension in PBS'A'. Soluble antigen extracts were prepared from SV5-infected BHK cells, as previously described (Randall et al,1987). These extracts were then mixed with the solid matrix-antibody complexes (20 $\mu$ l of a 10% w/v solid matrix-antibody complex per  $2 \times 10^8$  cell equivalents of antigen extract) for 4-6 h at 4°C. The resulting solid matrix-antibody-antigen (SMAA) complexes were washed three times by centrifugation and resuspension in immune precipitation buffer (20mM Tris-HCl, 5mM EDTA, 0.5% NP40, 0.1% SDS, 0.65M NaCl) and twice in PBS'A'. Mice were immunised twice intraperitoneally with 2-5 $\mu$ g of NP, M or HN SV5 protein attached to SMAA complexes, as 200 $\mu$ l aliquots of 0.5% w/v suspension of the SMAA complex, with a gap of 3-4 weeks between the first and second immunisations.

#### 4.2.7. X-irradiation of mice.

Six to eight week old Balb/c mice were exposed to 5 Gy whole body X-irradiation prior to infection with SV5. The effectiveness of irradiation was assessed by examining the ability of isolated splenocytes to respond to the B cell mitogen, lipopolysaccharide (LPS), and the T cell mitogen, concanavalin A (con.A) (R.E. Randall & D.F. Young). Immediately following X-irradiation of mice, their splenocytes failed to respond to either mitogen, and 10 days post-irradiation, the spleens had atrophied to such an extent that only  $2-4 \times 10^6$  splenocytes could be isolated per spleen, compared to  $1-2 \times 10^8$  from a normal spleen. The few splenocytes isolated from irradiated mice at this time showed some activation by con.A but none by LPS. However, 10 weeks post-irradiation, the mice had recovered to the extent that  $2-4 \times 10^7$  cells could be isolated per spleen, and the splenocytes proliferated in the presence of both con.A and LPS (R.E. Randall & D.F. Young, unpublished observations). Infection, immunisation and sacrifice of mice were carried out by D. Young and R.E. Randall.

### 4.3. RESULTS.

#### 4.3.1. Time course of SV5 infection in mouse lungs.

Mice were infected intranasally (IN) with SV5 and sacrificed at 5, 8, 12, 15 and 19 days post-infection (p.i.). Lung sections were cut and the relative amounts of viral RNA present within infected lung tissue was assessed by *in situ* hybridisation using the single-stranded M13 probe 4, specific for the SV5 HN gene. In all experiments, SV5-infected and uninfected Vero cell monolayers were used as positive and negative probe controls (not shown), and experiments were carried out in duplicate to minimise variation between mice.

Following hybridisation, lung sections were exposed to nuclear emulsion. After development, positive hybridisation to viral RNA was indicated by clusters of silver grains found in foci throughout the lung (Figure 4.2.). The amount of virus was maximal at 5 days post-infection (p.i.). By 8 days p.i. viral RNA was still detectable throughout the lung but the foci of silver grains were smaller and less numerous. By 12 days p.i., no positive hybridisation was detectable above the background hybridisation levels seen in uninfected mice (Figure 4.2.). No evidence was seen for the presence of virus at 15 and 19 days p.i. (not shown).

The presence of virus in infected lungs was also assessed by immunofluorescent detection of the P protein and gave comparable results to the detection of viral RNA, with levels of viral protein maximal at 5 days p.i. (not shown).

#### 4.3.2. Morphology of infected lungs.

Inflation of the lungs by injection of embedding fluid prior to freezing resulted in maintenance of the normal physical structure of the lungs, with alveoli clearly visible, and allowed preservation of the fine structure of the tissue throughout subsequent sectioning, fixation and hybridisation steps. This allowed differences in the morphology of uninfected and infected mouse lungs to be analysed.



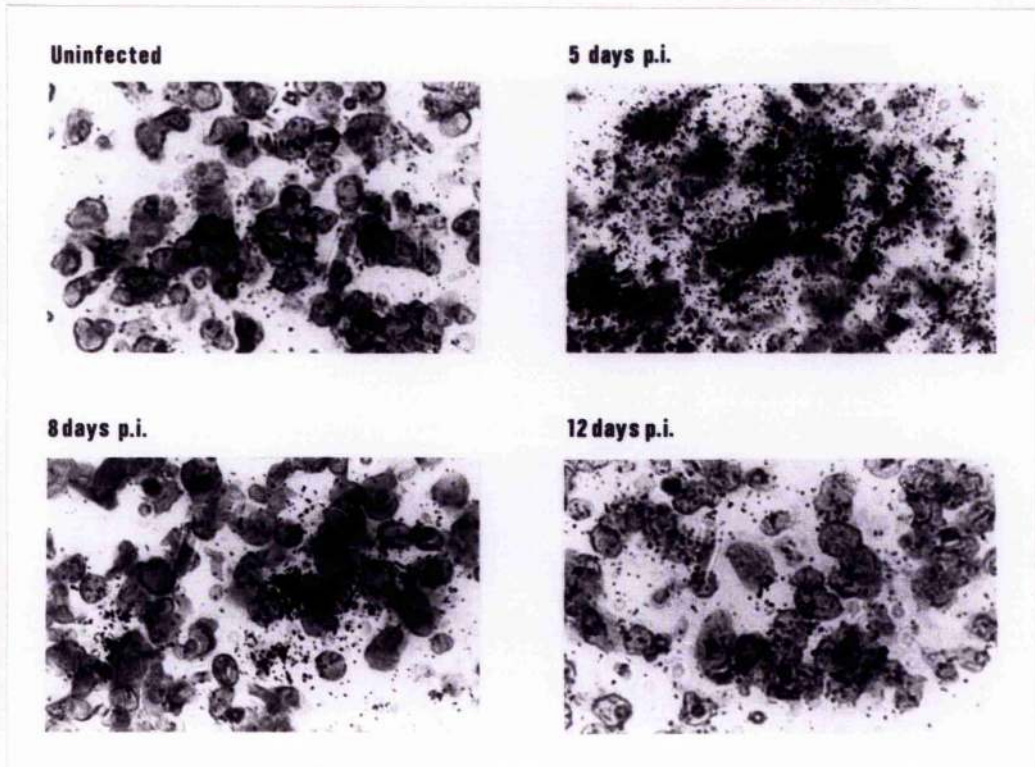


Figure 4.2. Detection of SV5 in mouse lungs by *in situ* hybridisation.

Mice were infected intranasally with SV5 and sacrificed at various times post-infection. The presence of SV5 RNA in lung sections was detected by hybridisation using an M13 DNA probe specific for the viral HN gene. Sections were exposed to nuclear emulsion for 2 weeks and the presence of virus indicated by foci of silver grains. Levels of viral RNA were maximal at 5 days p.i. By 12 days p.i. no RNA was detectable above background levels of hybridisation (uninfected lung). Stained with haematoxylin and eosin. Magnification x600.

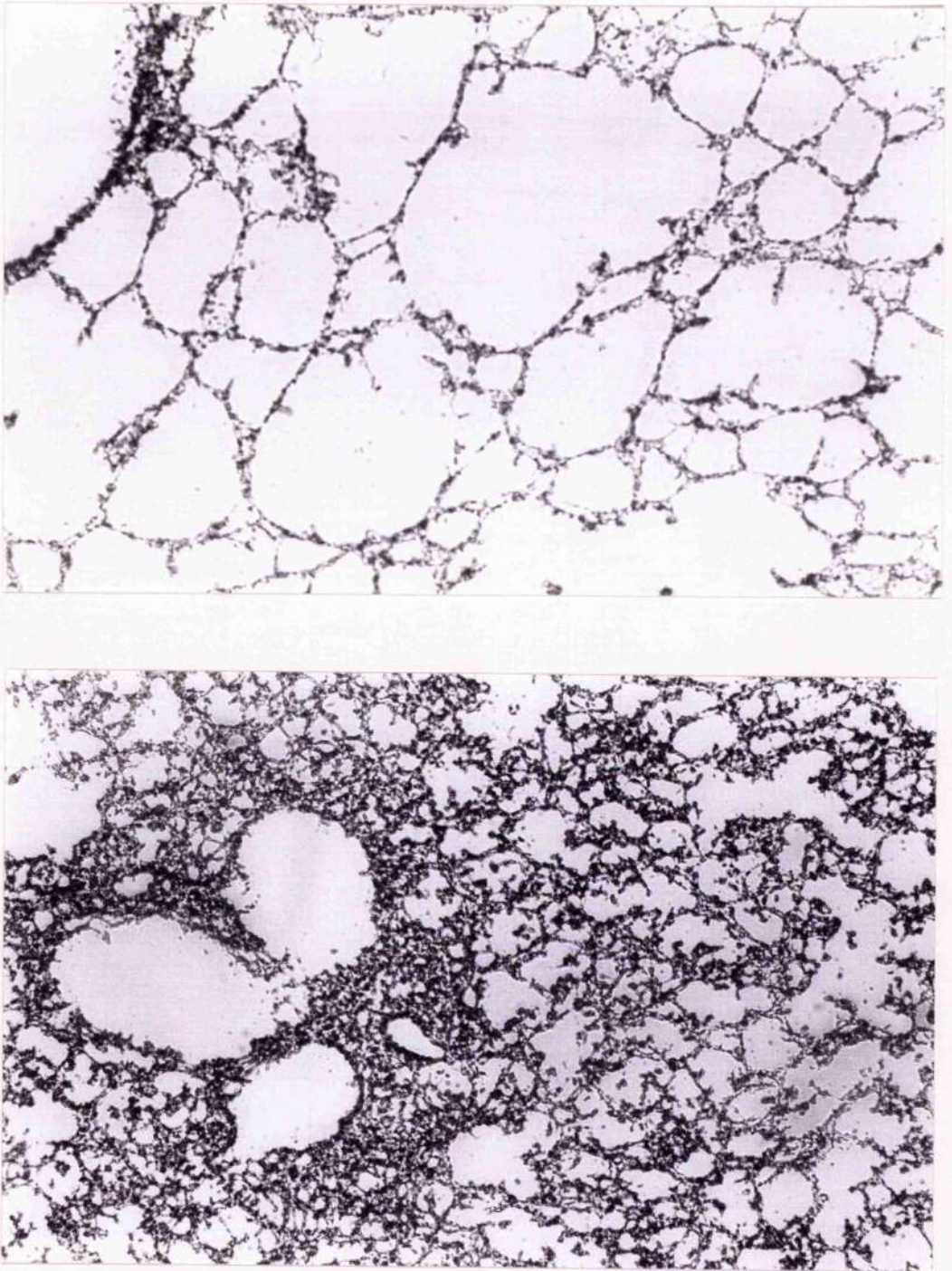
Lungs which had been infected with virus underwent morphological changes which resulted in areas of heavy congestion (Figure 4.3.). Indirect immunofluorescent staining was carried out using monoclonal antibodies specific for cells of the immune system of the mouse and showed that these areas of congestion were caused mainly as a result of infiltration of the lungs by macrophages and cytotoxic (CD8<sup>+</sup>) and helper (CD4<sup>+</sup>) T lymphocytes. Thus, at 5 days post-infection, large numbers of macrophages were readily detected in congested areas of the lungs (Figure 4.4.) compared with the low numbers of resting macrophages detected in uninfected lungs (not shown). CD8<sup>+</sup> and CD4<sup>+</sup> T-cells were undetectable in uninfected lungs, but were shown to be present in infected lungs, again concentrated in the congested areas, although the numbers detected were less numerous than the macrophages (Figure 4.4.).

The techniques of *in situ* hybridisation and immunofluorescence for detection of SV5 RNA and protein respectively allowed correlation of the distribution of virus with the morphological changes observed in infected lungs. The presence of virus, as detected by either method, was found to correlate with areas of congestion, and very little RNA or protein could be detected in areas of the lung where there was no congestion (not shown).

#### **4.3.3. Protection of mice by immunisation with purified viral proteins.**

'Fixed' and killed suspensions of Staph.A were saturated with monoclonal antibodies specific for the HN, NP and M proteins of SV5. These solid matrix-antibody complexes were then used to purify the appropriate protein from soluble antigen extracts of SV5-infected BHK cells. This purification step resulted in the virus proteins being purified to near homogeneity, as assessed by SDS-PAGE (Randall et al, 1988). Mice were immunised twice intraperitoneally with these SMAA complexes and challenged by infection with SV5, three to four weeks after the second

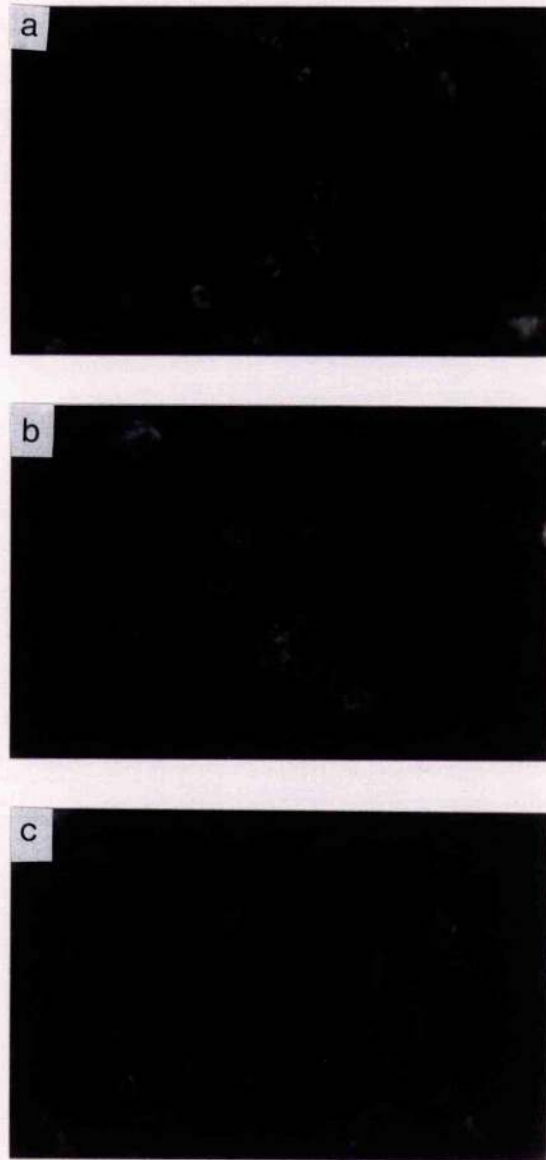




**Figure 4.3. Morphology of uninfected and infected mouse lungs.**

Sections were cut from inflated lungs of uninfected and infected mice. Infected lungs (bottom) showed large areas of congestion compared to uninfected lungs (top) where alveoli were clearly visible.

Stained with haematoxylin and eosin. Magnification x150.



**Figure 4.4. Detection of mouse macrophages and T-cells in infected lungs.**

Mice were infected with SV5 and sacrificed at 5 days post-infection. Lung sections were fixed with 5% formaldehyde, 2% sucrose. The presence of (a) macrophages ( $m\phi$ ), (b) cytotoxic T-cells ( $CD8^+$ ) and (c) helper T-cells ( $CD4^+$ ) was detected by indirect immunofluorescence with a rhodamine-conjugated goat anti-mouse polyclonal antibody, following binding of mouse cell-specific monoclonal antibody.

Magnification  $\times 300$ .



immunisation. Five days p.i., the mice were sacrificed and the relative levels of viral RNA or protein in their lungs was estimated by *in situ* hybridisation and immunofluorescence respectively. Mice immunised with Staph.A only served as a positive control for normal levels of virus replication and uninfected mice as a negative control.

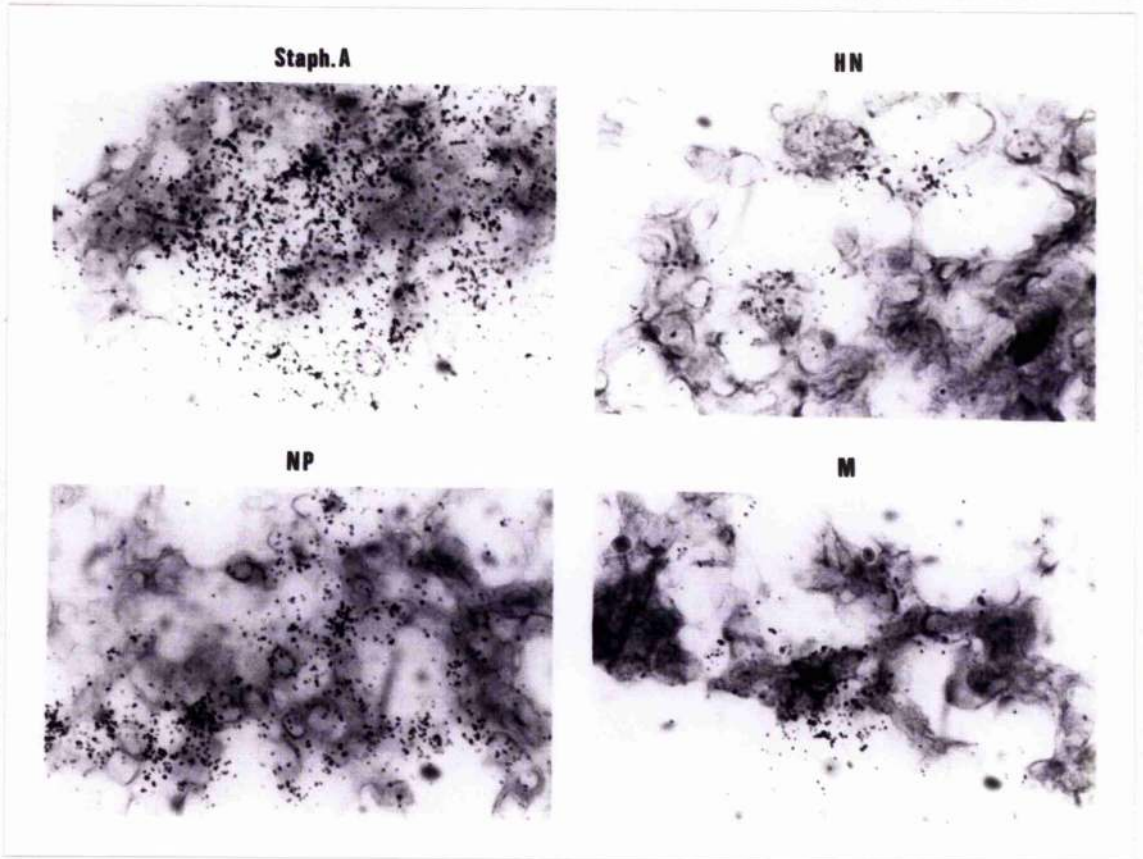
All comparisons of relative amounts of RNA and protein were made between heavily congested areas of the infected lungs, since the presence of virus was previously shown to correlate with these areas (Section 4.3.2.). This ensured that an apparent reduction in the level of virus in mice which had been immunised was not due to the absence of virus *per se* in that area of the lung.

The results obtained by both *in situ* hybridisation (Figure 4.5.) and immunofluorescence (Figure 4.6.) were found to be directly comparable. Thus, mice immunised with either external (HN) or internal (NP and M) proteins had reduced levels of viral RNA and P protein compared to control mice immunised with Staph.A only. Immunisation with the HN protein appeared to afford the highest degree of protection, with very little virus present throughout the lung. However, a significant degree of protection was also observed following immunisation with the NP and M proteins.

A comparison of the lung morphology between mice which had been immunised with viral proteins and those immunised with Staph.A showed that areas of inflammation were still present in immunised lungs. However, the level of congestion appeared to be somewhat reduced compared to the Staph.A-immunised controls (not shown).

#### **4.3.4. Persistence of SV5 in X-irradiated mice.**

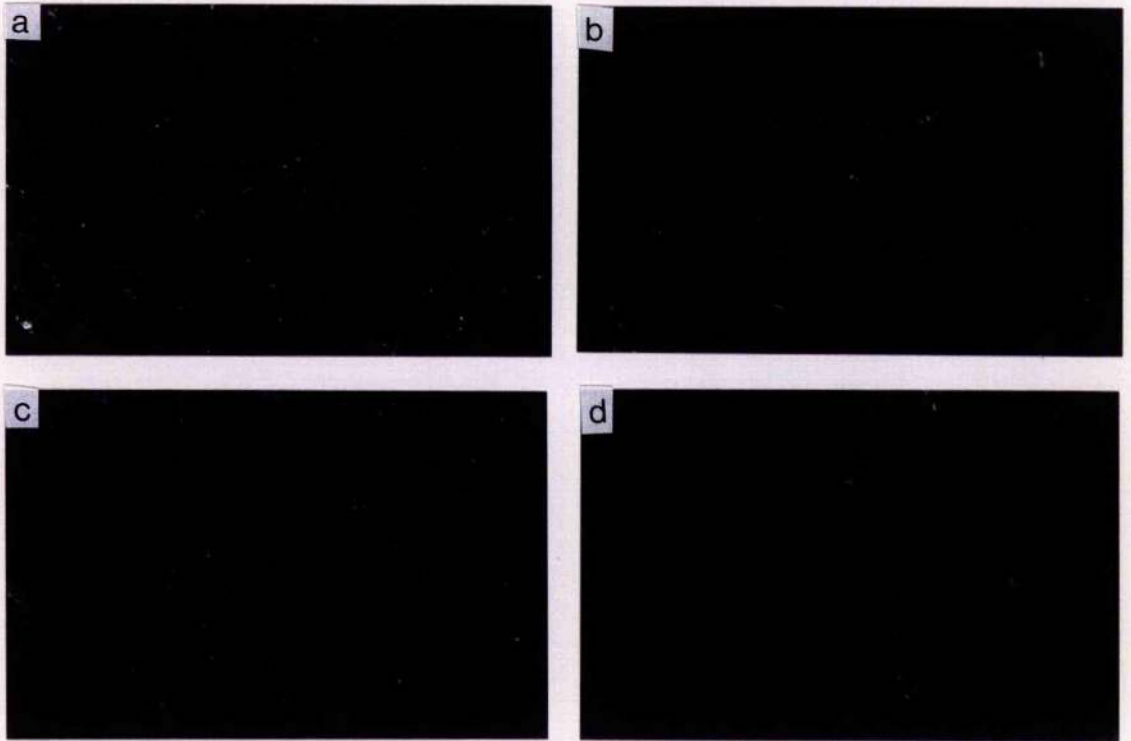
Mice made immunodeficient by X-irradiation were immediately infected with SV5. At 5, 10, 14 and 19 days p.i., mice were sacrificed and the relative amounts of SV5 RNA in the lungs was analysed. These were compared to the level of RNA detected



**Figure 4.5.** Detection of SV5 in the lungs of immunised mice by *in situ* hybridisation.

Mice were immunised with Staph.A-antibody-antigen complexes of NP, M and HN proteins of SV5, followed by infection with SV5. Mice were sacrificed at 5 days post-infection and the presence of SV5 RNA was detected with an M13 probe specific for the viral HN gene. Slides were exposed to nuclear emulsion for 2 weeks. A reduction in the level of hybridisation to viral RNA was seen in immunised mice compared to the control Staph.A-immunised mice. No hybridisation was seen in uninfected mice (not shown).

Stained with haematoxylin and eosin. Magnification x600.

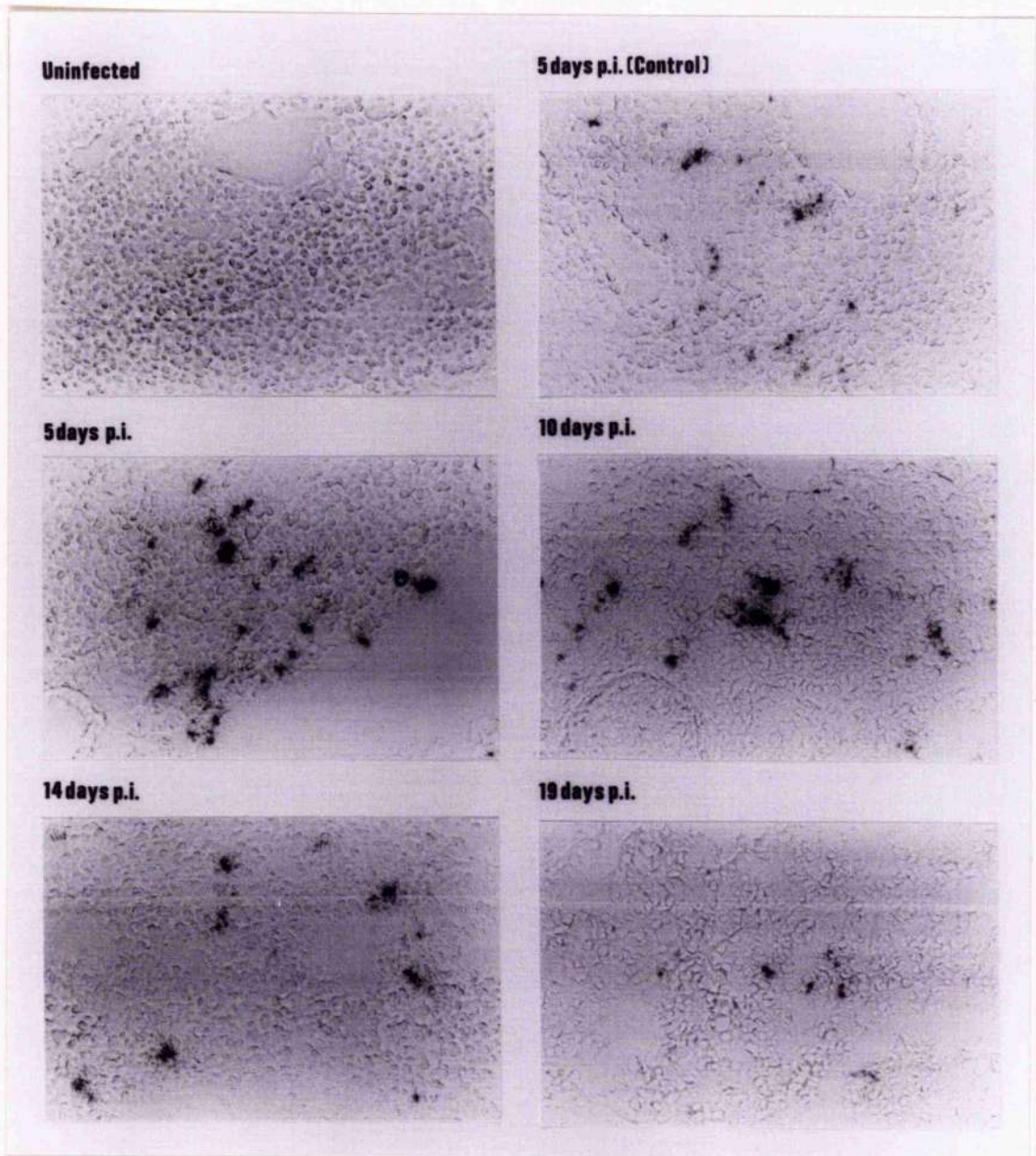


**Figure 4.6. Detection of SV5 in immunised mouse lungs by immunofluorescence.**

Mice were immunised with Staph.A-antibody-antigen complexes of NP, M and HN proteins of SV5, followed by infection with SV5. Mice were sacrificed at 5 days p.i. and the presence of virus was detected by direct immunofluorescence with a mixture of monoclonal antibodies raised against the P protein of SV5 and labelled with FITC. (a) Staph.A only, (b) NP, (c) M, (d) HN. A reduction in the level of virus-specific fluorescence was seen in mice which had been immunised with viral proteins, compared to the control Staph.A-immunised mice. No specific fluorescence was seen in uninfected mice (not shown).

Magnification x150.





**Figure 4.7. Detection of SV5 RNA in X-irradiated mouse lungs.**

Mice were X-irradiated, infected with SV5 and sacrificed at various times post-infection. The presence of SV5 was detected using an M13 probe specific for the viral HN gene. Sections were exposed to nuclear emulsion for 2 weeks. Uninfected mice were used as a negative control and unirradiated infected mice as a positive control for normal levels of virus. Viral RNA could be detected up to 19 days p.i. Sections were not stained. Magnification x150.



in immunocompetent mice at 5 days p.i., the time of maximum replication. The effectiveness of X-irradiation was shown by the greatly reduced numbers of splenocytes which could be isolated from the spleens of irradiated mice compared to unirradiated mice, and the inability of these splenocytes to respond to B and T cell mitogens (R.E. Randall & D.F. Young).

In immunodeficient mice, viral replication was prolonged or persistent. Thus, in these animals viral RNA was still detectable at times post-infection when the virus would have been completely cleared in immunocompetent mice (Figure 4.7.). Clearance of virus from the lungs occurred gradually as the immune system of the mouse recovered. However, low levels of virus were still detectable at 19 days p.i. (Figure 4.7.), compared to complete clearance by 12 days p.i. in immunocompetent mice (Figure 4.2.). These results therefore show that a persistent infection of SV5 can be established in mice in the absence of an effective host immune response.

#### 4.4. DISCUSSION.

A major aim of the paramyxovirus group at St. Andrews has been to develop an animal model for studies of virus-host interactions during replication of the paramyxovirus, SV5. Here, I have demonstrated the use of *in situ* hybridisation as a means of analysis of the effects on viral replication of experimental perturbation of the host's immune system. The use of animal models for such studies allows direct manipulation of the host, enabling identification of the specific immune mechanisms involved in clearance and protection from viral infections. Most studies of the immune response to viral infection have analysed the effects of perturbation of the hosts immune system on viral replication by measurements of infectious virus titres. However, the use of techniques such as *in situ* hybridisation for detection of viral nucleic acids, together with methods such as immunofluorescence and Western blotting, allow more direct comparisons *in vivo*. In addition, direct detection *in situ* allows the presence and distribution of virus to be correlated with morphological changes in the infected tissue. These techniques have the advantage of direct detection of viral components, rather than infectious virus, since it has been reported that the presence of neutralising antibodies during extraction of lungs may affect accurate determination of viral titres (Randall & Young, 1988). In addition, during the establishment of persistent infections, little infectious virus may be produced.

It has previously been shown that mice can be infected with SV5, with titres of infectious virus reaching maximal levels at 4-5 days following intranasal infection (Chang & Hsiung, 1965). The use of *in situ* hybridisation to detect viral RNA gave comparable results, with levels of virus nucleic acid maximal at 5 days post-infection, and thereafter rapidly declining, such that by 12 days p.i., no viral RNA was detectable. It has been shown that an increase in the level of viral RNA and protein occurs between 1 and 5 days p.i. (Randall et al, 1988), indicating that viral replication is occurring, rather than simply detection of input virus. Examination of the morphology of infected lungs showed large areas of congestion or inflammation,

caused by an influx of macrophages and cytotoxic and helper T-lymphocytes, which was not seen in uninfected lungs. Areas of inflammation correlated completely with the presence of virus, as detected by *in situ* hybridisation or immunofluorescence. This system was further developed to determine its possible use as a model for studies of the host factors involved in protection against SV5 infection.

The degree of protection to SV5 infection induced in mice following immunisation with various purified SV5 proteins, was analysed by a comparison of RNA and protein levels in immunised and non-immunised mice at 5 days p.i., the period of maximum replication in unimmunised mice. Since the presence of virus in infected lungs was previously shown to correlate completely with areas of inflammation, comparisons of virus levels were made only between these areas, to ensure that an apparent reduction in the amount of viral RNA or protein was not due to the absence of virus in that area of the lung. Both external (HN) and internal (NP and M) proteins were found to be effective in eliciting an immune response which enhanced the speed of clearance of virus. This was indicated by a decrease in the level of hybridisation to viral RNA and fluorescent detection of protein. This is in agreement with the results of Randall et al (1988) in which a decrease in the level of viral proteins was observed by Western blot analysis. These authors also reported the presence of high levels of neutralising antibodies in serum but there was no apparent correlation between the presence of antibodies and the degree of protection observed following immunisation. However, the presence of SV5-specific cytotoxic T-lymphocytes was demonstrated in the spleens of infected mice, both immunised and non-immunised, but not in uninfected mice (Randall et al, 1988). Clearance of SV5 in infected mice therefore appears to be dependent mainly on a cell-mediated immune response. Since areas of inflammation were still present in immunised mice, the effect of immunisation is presumably to enhance clearance of virus by inducing the rapid production of primed T-cells, rather than prevent infection of cells.

The ability to establish a persistent infection in mice made immunodeficient by

X-irradiation was analysed by a comparison of viral RNA levels in the lungs of infected mice at various times p.i. A prolonged or persistent infection was seen to occur in such immunodeficient mice, with viral RNA still detectable at 19 days p.i. compared to complete clearance of virus by 12 days p.i. in immunocompetent mice. Western blot analysis has shown that viral proteins are also detectable at 19 days p.i. but that virus is cleared by 28 days p.i. (Young et al, 1990). Infectious virus was detectable up to 19 days p.i., but titres were relatively low, never reaching the level of input virus (Young et al, 1990). Since a significant increase was seen in the amounts of viral RNA and protein, this indicates that a persistent infection without significant production of infectious virus is occurring.

X-irradiation destroys all dividing cells, including those of the immune system, and hence the host is unable to mount an effective immune response. However, a reduction in the level of viral RNA was eventually observed in immunodeficient mice, presumably due to the gradual recovery of the immune system of the mouse, which has been shown to occur within 2-3 weeks post-irradiation (Anderson & Standefer, 1983). Thus, a prolonged persistent infection can be induced in immunodeficient mice compared to the rapid clearance of virus seen in immunocompetent mice.

Subsequent studies in this model of persistent infection have demonstrated that CD8<sup>+</sup> cells are primarily responsible for clearance of SV5 infection in mice (Young et al, 1990). The ability of selected T-cell subpopulations to enhance clearance of persistent virus by cell transfer experiments was used to identify the major populations involved in clearance. Thus, transfer of SV5-primed Class I-restricted CD8<sup>+</sup> lymphocytes resulted in rapid clearance of virus, as assessed by Western blot analysis (Young et al, 1990). A small degree of protection was observed following transfer of Class II-restricted CD4<sup>+</sup> lymphocytes. However, this may have been due to contamination of the cell population with CD8<sup>+</sup> cells. The predominant cell type involved in protection and clearance of SV5 infection in mice therefore appears to be

CD8<sup>+</sup> T-lymphocytes. This is in agreement with earlier studies of the protection induced by immunisation with SV5 proteins (Randall et al,1988; Randall & Young,1988).

The presence of neutralising antibodies in serum has been shown to have little effect on clearance of virus in immunodeficient mice (Randall et al,1988; Young et al,1990), in contrast to reports of the protective effect of neutralising antibodies against infection with other paramyxoviruses (Taylor et al,1984; Giraudon & Wild,1985; Wolinsky et al,1985; Love et al,1986). This difference could possibly be explained by the fact that infection of immunodeficient mice with SV5 appears to result in a non-productive persistent infection, in which clearance of virus-infected cells occurs primarily by a T-cell dependent mechanism. In contrast, studies of experimental infection with other paramyxoviruses have resulted in productive infections in which the presence of neutralising antibodies may play a protective role by preventing spread of infectious virus within the tissue. Thus, a persistent SV5 infection can be maintained in the presence of high levels of neutralising antibodies, as seen for example in persistent infection of measles virus, resulting in subacute sclerosing panencephalitis. The ability to directly detect viral RNA and proteins in such a system, where high levels of infectious virus are not produced, should allow more detailed analysis of the role of the immune response to such viral infections.



**CHAPTER FIVE.**

**Detection of measles virus in  
two chronic human diseases.**

## 5.1. INTRODUCTION.

### 5.1.1. Persistent infections by paramyxoviruses.

It is now well recognised that paramyxoviruses can cause persistent infections with little or no production of infectious virus, which may result in chronic disease symptoms. Such infections are referred to as slow or chronic infections due to the long incubation time of months to years before onset of clinical symptoms, and differ from the latent infections seen in herpesviruses and retroviruses, in which virus replication is specifically restricted. The recognition that viruses can persist in the absence of detectable levels of infectious virus or even virus protein, has reawakened interest in the search for viruses in a number of chronic diseases of unknown aetiology, such as multiple sclerosis. The ability of *in situ* hybridisation to detect low levels of viral nucleic acids in such situations, where only very small numbers of cells are likely to harbour the virus, should provide an invaluable technique for detection of putative aetiological agents.

Possible mechanisms of persistence have been studied in most detail for measles virus, the aetiological agent of the chronic human disease subacute sclerosing panencephalitis (SSPE). However, a number of paramyxoviruses, including measles virus, have been implicated in several other chronic human diseases which are discussed in more detail below. In this chapter, the ability to detect measles virus RNA by *in situ* hybridisation has been investigated in two diseases, SSPE and Paget's disease of bone.

### 5.1.2. Mechanisms of persistence.

The mechanisms of persistence *in vivo* have yet to be determined and are likely to involve complex interactions between virus and host. Two aspects need to be addressed in such studies. Firstly, the molecular mechanisms involved in virus-host cell interactions which lead to the establishment of persistence, and secondly, the role played by the immune system of the host in allowing the establishment of a

persistent infection while being capable of controlling acute infection. Reconstructions of the situation *in vivo* are obviously difficult although there are animal models available for some human diseases. Many studies of persistence have therefore concentrated on the establishment of persistence *in vitro* and have suggested a number of possible mechanisms.

The host immune response to infection may itself result in persistence. Antigenic modulation has been demonstrated *in vitro*, where capping of viral surface proteins by antibodies, followed by shedding of these antigen-antibody complexes results in cells devoid of surface antigens which therefore escape lysis by antibody plus complement (Joseph & Oldstone, 1974; 1975; Oldstone & Tishon, 1978; Fujinami et al, 1981). In most areas of the body *in vivo*, where complement is readily available, this may not play a significant role, but in areas where complement is absent, such as the central nervous system (CNS), it may be important. The possible importance of the humoral immune response is also suggested by the fact that over 50% of SSPE patients have contracted measles virus infection at a very early age when maternal antibodies may still have been present (Pettay et al, 1971). Similarly, the administration of measles specific antibodies following infection in mice has been shown to enhance persistence *in vivo* (Rammohan et al, 1982).

A number of viruses are known to cause direct functional impairment of the immune system. For example, measles virus directly affects the immune response by infection of lymphocytes (Casali et al, 1984; McChesney et al, 1986). Such viruses may be able to specifically alter the immune response raised against them and hence escape immune surveillance and persist in the host (McChesney & Oldstone, 1987; Oldstone, 1989).

Virus-induced mechanisms of persistence may include the production of defective-interfering (DI) particles which interfere with replication of infectious virus and have been shown to cause the establishment of persistence *in vitro* by

passage of undiluted virus stock in which such DI particles rapidly accumulate (Holland et al,1976; Rima et al,1977; Roux & Holland,1979).

The selection of antigenically modified distinct viral mutants is generally favoured in the presence of an immune response, since such mutants can then escape detection by the immune system. The low fidelity rate of RNA polymerase may play an important role in the rapid generation of such mutants during the establishment of persistence (Holland et al,1982; Steinhauer & Holland,1987; Cattaneo et al,1988). It has also been suggested that the double-stranded RNA unwinding/modifying activity of the host cell may enhance mutation rate in RNA viruses (Bass & Weintraub,1988; Lamb & Dreyfuss,1989). Rapid evolution of viral mutants has been demonstrated for a number of paramyxoviruses *in vitro* during the establishment of persistence, including Newcastle disease virus (NDV) (Preble & Youngner,1973a,b), Sendai virus (Kimura et al,1975), mumps (Truant & Hallum,1977) and measles virus (Knight et al,1972; Haspel et al,1975; Minagawa et al,1976). However, the importance of this *in vivo* has yet to be established.

Lastly, interferon has been suggested as a host-specific mechanism of induction of persistence (Carrigan & Kabacoff,1987). Interferon is the name given to a group of cell-specific anti-viral proteins. Under normal conditions, infection of a cell with a virus induces the production of interferon which renders surrounding non-infected cells more resistant to infection and hence helps to halt the spread of infection. The anti-viral state is effective for several days and forms one of the first lines of defence against viral infection until a full immune response is established. Interferon reduces viral multiplication by, amongst other things, interfering with the ability of viral mRNAs to be translated. Thus, in the presence of interferon, infection is aborted and the cells are spared from destruction by the cytopathogenicity of the virus. However, the virus may not be eliminated from the cell and therefore interferon has been postulated to enhance the establishment of

persistence (Ito et al,1975; Rima & Martin,1976; Friedman & Ramseur,1979). If it does play a role, however, it is likely to be a minor one, since persistent infections are readily established in cell lines which do not produce interferon, such as Veros and BHK21s (Youngner & Preble,1980).

### **5.1.3. Chronic human diseases with proposed paramyxovirus aetiology.**

#### **5.1.3.1. Subacute sclerosing panencephalitis.**

Subacute sclerosing panencephalitis (SSPE) is a fatal, slowly progressing inflammatory disease of the central nervous system (CNS), affecting mainly children and young adults. The involvement of viruses in the disease was first proposed by Dawson in 1933, following observations of intranuclear inclusions in brain cells of patients. These inclusions were later shown to contain paramyxovirus nucleocapsid structures. Evidence for the involvement of measles virus was supported by the detection of very high measles-specific antibody titres in the serum and cerebrospinal fluid (CSF) of SSPE patients (Connolly et al, 1967) and by fluorescent studies in which measles virus antigens were detected in neurons and glial cells (ter Meulen et al,1967). More recently, measles virus RNA has been demonstrated in brain and lymphatic tissue by *in situ* hybridisation (Haase et al,1981b; Fournier et al,1985; Shapsak et al,1985). The pathogenesis of SSPE has yet to be explained. However, the features of the disease differ from acute measles virus infection and measles encephalitis (ter Meulen & Hall, 1978), and additional host or viral factors are assumed to be involved since the rarity of occurrence of SSPE ( $1/10^6$  acute MV infections) cannot be correlated with the ubiquitous nature of measles virus infections. Progression of SSPE can be broadly divided into three stages - intellectual deterioration and psychological disturbances, neurological and motor dysfunctions, and finally a progressive cerebral degeneration and decortication. However, the disease is highly variable lasting from months to years, and remissions are common (Risk et al, 1978). Neuropathologically, the most



characteristic change seen is the presence of intranuclear inclusion bodies in neurons, astrocytes and oligodendroglial cells, first described by Cowdry (1934), which contain paramyxovirus nucleocapsid structures (Tellez-Nagel & Harter, 1966).

A consistent diagnostic characteristic of SSPE is the presence of exceptionally high measles virus antibody titres in both serum and CSF, which have not been observed to such an extent with other measles virus-associated diseases, such as measles encephalitis. This is mainly the result of an increase in IgG antibodies, which exhibit restricted banding on electrophoresis, indicating an oligoclonal population (Vandvik, 1973) and which can be removed by absorption with highly concentrated, purified measles virus preparations (Vandvik et al, 1976). The low ratio of serum:CSF antibody levels found (Connolly, 1968; Salmi et al, 1972) indicates local production of measles virus antibodies with a state of hyperimmunisation of the CNS. This oligoclonal hyperimmune response appears to be a general characteristic of viral infections of the CNS and may be able to favour production of viral mutants, since antibodies which are directed against specific determinants of the virus only would increase the probability of a mutant being able to escape neutralisation.

The overall levels of both viral and mRNAs are greatly reduced in persistently-infected SSPE cells compared to productively-infected cells (Haase et al, 1985b). It has been suggested by various workers that defective expression of the matrix protein could be a possible mechanism of measles virus persistence preventing assembly of virions. Initial immunochemical studies failed to detect M protein (Hall et al, 1979; Hall & Choppin, 1979). However, later studies using monoclonal antibodies have detected M protein in some patients (Norrby et al, 1985; Baczko et al, 1986). The H and F proteins are also sometimes undetectable, indicating that defective expression of more than one protein may be involved (Wechsler & Fields, 1978; Johnson et al, 1982; Baczko et al, 1986; Cattaneo et al, 1988). It is

interesting that these proteins are involved in assembly of infectious virus particles and not in replication or transcription.

The importance of defects in the M and other proteins remains unresolved. However mRNA for the M protein has been detected by *in situ* hybridisation indicating that the gene is present, although it may be mutated (Haase et al,1981a; Shapsak et al,1985; Haase et al,1985b). However, although transcription is reduced, defects may also occur at the translational level since bicistronic P-M RNA has been detected (Cattaneo et al,1986) and *in vitro* translational studies have indicated that not all mRNAs are translated efficiently (Baczko et al,1986). It has also been reported that the levels of H and F mRNA are reduced relative to other genes (Baczko et al,1986).

Discrepancies in the detection of various viral proteins may be partially explained by fluctuations during the course of the disease. For example, it has been shown that expression of the M protein is reduced at later times in the course of the disease (Haase et al,1985b; Ilonen et al,1987; Ohuchi et al,1987). It is apparent that the establishment of persistence *in vivo* is complex and appears to involve a number of interacting mechanisms. A key factor appears to be the rapid accumulation of non-specific mutations which can occur in a number of genes. These may result in a variety of molecular mechanisms of persistence indicated by the isolation of measles virus isolates with different replication defects from different patients, and even from different regions of the same patient (Baczko et al,1986). The exact mechanism of pathogenesis remains unresolved.

#### 6.1.3.2. Paget's disease of bone.

Paget's disease of bone (Osteitis deformans) was first described by Paget in 1877. It is a chronic human bone disorder, affecting approximately 4% of the population over 70 years of age (Collins,1956; Hamdy,1981) and is particularly common in the North West of England, with up to 5-8% of the elderly affected in

some areas (Barker et al,1977; 1980). Although frequently asymptomatic, it is often associated with painful and deformed bones, and may result in fractures and nerve compressions. The bone has an abnormally irregular structure, with increased activities of both the osteoclast and osteoblast cells, accompanied by invasion of the medullary space by fibrotic tissue which gradually replaces the haematogenous marrow (Krane,1977; Hamdy,1981). Treatment for the disease is now available in the form of 3-amino-hydroxypropylidene-1,1-bisphosphonate (APD) which is incorporated into newly-formed bone. This is then resorbed by the overactive osteoclasts resulting in their death. This treatment results in remissions in over 90% of cases, often with a remarkable return towards the normal bone morphology, with dramatic reductions in osteoclast numbers and area of active bone surfaces (Cantrill et al,1986; 1988).

The osteoclasts are large multinuclear cells, formed by fusion of precursor cells, which are responsible for the reabsorption of bone. They are normally found lying against the bone surfaces of resorption cavities (Krane,1977; Rebel et al,1980b; Hamdy,1981) and have a specialised membrane, the ruffled border, which is involved in dissolution of the bone matrix and transport of the matrix components through the cell with the ultimate release of calcium and other components into the vascular spaces (Osdoby et al,1987). Activity of the osteoclast cells is closely linked to the activity of the osteoblast cells (Fallon,1983; McSheehy & Chambers,1986), which are responsible for the formation of new bone. It is generally accepted that osteoblasts arise from differentiation of uncommitted mesenchymal cells (Hall,1972) and that osteoclasts originate from blood-borne or marrow haematopoietic cells related to the monocyte-macrophage family (Walker,1975). It is thought that resident or circulating mononuclear cells are attracted towards bone by chemotaxis, then attach, fuse and differentiate into osteoclast cells (Osdoby et al,1987).

Paget's disease of bone has for many years been believed to be the result of a

slow virus infection of the osteoclasts (Singer,1980), resulting in overactive and uncontrolled resorption of bone. It has been suggested that the disease arises from a single blood-borne infection of osteoclast precursor cells, which then settle in the bone (D. Anderson; personal communication). Infection of the osteoclast could result in abnormal control of resorption, for example, increased fusion resulting from infection would result in the abnormally large osteoclasts typical of Pagetic bone, in which the balance of control of resorption may be upset. Alternatively, the infected cell could be induced to produce more bone resorption promotion factors. Increased osteoblastic activity is also observed in Pagetic bone but is most probably the result of the normal interaction between osteoclast and osteoblast activity.

There is strong evidence to suggest that paramyxoviruses may be involved in the aetiology of Paget's disease of bone. Initial indications came from ultrastructural analysis of osteoclasts in affected bone, which showed characteristic inclusion bodies in the nucleus and cytoplasm (Mills & Singer,1976; Rebel et al,1977; Harvey et al,1982) containing filaments identical to paramyxovirus nucleocapsids. These inclusions were specific to the osteoclasts (Harvey et al,1982) and a correlation was found between the percentage of nuclei containing inclusions and the severity of the disease (Harvey et al,1982).

Immunocytochemical studies have demonstrated proteins from a number of paramyxoviruses in osteoclasts. Measles virus has been implicated most consistently (Basle et al,1979; 1985; 1987; Rebel et al,1980a; Mills et al,1982; 1984; Mills & Singer,1987) but proteins of other paramyxovirus have also been reported, including respiratory syncytial virus (RSV) (Mills et al,1981; 1982; 1984; Singer & Mills,1983; Basle et al,1985), parainfluenza virus type 3 (PF3) (Basle et al,1987) and simian virus 5 (SV5) (Basle et al,1985; 1987). Canine distemper virus (CDV) has been implicated by seroepidemiological evidence (O'Driscoll & Anderson,1985) but disputed by other workers (Hamill et al,1986). Recently the presence of MV RNA in Pagetic bone was detected by *in situ* hybridisation (Basle et

al,1986) but the presence of RNA of other paramyxoviruses has not yet been investigated by this method.

#### 6.1.3.3. Other diseases.

Recent studies have indicated that paramyxoviruses, in particular MV, may play an aetiological role in a number of other chronic human diseases.

MV has been implicated in the pathogenesis of autoimmune chronic active hepatitis (AICAH). Subclinical hepatitis has been reported to be frequently associated with MV infections (Gavish et al,1983) and high levels of MV-specific antibodies are found in AICAH patients, comparable to the levels found in SSPE patients (Triger et al,1972; Robertson et al,1987). These consist mainly of an IgM fraction (Christie & Hawkenes,1983) indicating continuous stimulation of the immune system, as in a persistent infection. The age of onset of AICAH has increased over the last 30 years and this increase has been linked to the introduction of the measles virus vaccine (Robertson et al,1989), adding further circumstantial evidence to the putative role of MV. Dot blot hybridisation has been used to detect MV RNA in a high proportion (70%) of the lymphocytes of AICAH patients (Robertson et al,1987), indicating that MV does persist in these patients. This however does not demonstrate a causal relationship, since MV may persist in the lymphocytes of people who have had a normal MV infection or have been vaccinated (Black,1988). Indeed, a recent study detected MV RNA using the polymerase chain reaction in the lymphocytes of 7/8 AICAH patients, but also in 4/6 controls (Kalland et al,1989), indicating that MV persistence may be a common phenomenon.

The presence of measles virus RNA has also recently been reported in the lymphocytes of a high proportion of patients suffering from the chronic autoimmune diseases systemic lupus erythematosus (SLE) (Robertson et al,1987; Andjaparidze et al,1989) and chronic glomerulonephritis (CGN) (Andjaparidze et al,1989). Thus, MV RNA was detected in 85% of SLE patients (28/34) and 59% of CGN



patients (40/68). The higher detection rate of MV in SLE patients correlated with a higher average MV antibody titre in these patients (Andjaparidze et al,1989). The possible role of measles virus in the aetiology of these diseases requires further investigation.

A number of viruses, in particular paramyxoviruses, have also been implicated in the aetiology of multiple sclerosis (MS), a chronic neurological disease characterised by inflammatory demyelinating lesions widely disseminated throughout the white matter of the CNS. These lesions are generally considered to be the result of an autoimmune reaction against myelin basic protein, the cause of which is unknown. However, much circumstantial evidence points to a viral aetiology, and paramyxoviruses, with their known ability to persist in the CNS, are strong candidates. In particular, measles virus has been consistently implicated. For example, elevated levels of MV antibodies are found in the serum and CSF (reviewed by Norrby,1978), although raised levels of antibodies have also been found for a number of other viruses such as mumps, parainfluenzaviruses 1 and 3, herpes simplex virus, varicella-zoster virus and vaccinia virus (reviewed by ter Meulen & Stephenson,1981; Russell,1983). MV RNA has also been detected by *in situ* hybridisation in some MS brain tissue (Haase et al,1981; 1985; Cosby et al,1989).

Further studies should help elucidate the possible role played by MV and other paramyxoviruses in these diseases.

## **5.2. MATERIALS AND METHODS.**

### **5.2.1. Preparation of brain sections from a subacute sclerosing panencephalitis patient.**

Blocks of fresh brain were obtained post-mortem from a patient with subacute sclerosing panencephalitis from Dr L. Cosby (Queens University, Belfast). Blocks were frozen in liquid nitrogen and 10µm sections cut onto silanated slides (see Appendix, Section V), air dried briefly and fixed with ethanol:acetic acid (3:1).

### **5.2.2. Preparation of sections from Paget's disease of bone tissue.**

Sections which had been prepared from biopsy material from five patients with Paget's disease of bone were obtained from Prof. D. Anderson (Manchester University). The bone had been decalcified, formaldehyde-fixed and paraffin-embedded, and the sections mounted on slides which had been treated with wood-glue. Sections were dewaxed by incubation in three changes of fresh xylene for 5 mins each at room temperature to remove all traces of paraffin. They were then washed twice in absolute alcohol for 5 mins each and air dried.

### **5.2.3. *In situ* hybridisation.**

*In situ* hybridisation was carried out as described in Section 3.2. Sections were pretreated with 0.2M HCl, digested with 1µg/ml proteinase K and dehydrated in graded alcohols. The high temperature step was omitted to reduce loss of sections from the slides. Hybridisation was carried out at 37°C for 24 hours.

The M13 probes (Z26, 4 & 112H) were used at a concentration of 3ng/ml, and the oligonucleotide probe 228924 at a concentration of 1ng/ml. Post-hybridisation washes included a high stringency wash at 60°C in 2X SSC. Slides were coated with nuclear emulsion and exposed at 4°C for varying lengths of time up to 2 months. Following development, slides were stained with haematoxylin and eosin.

#### **5.2.4. Extraction of RNA from paraffin-embedded tissue samples.**

RNA was extracted from the formaldehyde-fixed, paraffin-embedded sections of Paget's bone tissue using a modified DNA extraction protocol (Jackson et al,1989). Five sections from each patient were dewaxed in fresh xylene at 37°C for 20 mins, washed once with xylene at room temperature for 2 mins and rehydrated with graded alcohols. The tissue was scraped off the slide into 100-200µl of digestion buffer (100mM NaCl, 10mM Tris-HCl pH 8.0, 25mM EDTA, 0.5% SDS) containing 0.1mg/ml Proteinase K (BRL), and incubated at 37°C for 5 days. Nucleic acids were purified by phenol:chloroform extraction, then ethanol-precipitated and resuspended in 30µl RNase-free water.

#### **5.2.5. Polymerase chain reaction (PCR).**

Amplification of DNA was carried out in a total volume of 50µl, containing 50pmoles of each primer, 5µl 10X PCR buffer (10X = 500mM KCl, 100mM Tris-HCl pH8.3, 15mM MgCl<sub>2</sub>, 0.1% (w/v) gelatin, 0.1% Triton X), 8µl dNTP mix (1.25mM each dATP, dCTP, dGTP, dTTP), 100-200ng genomic DNA or 1-2ng plasmid DNA and 2.5U Taq DNA polymerase (Promega). The volume was made up with HPLC-purified water and the sample overlayed with 40µl liquid paraffin to prevent evaporation. The samples were incubated in a dry block thermal cycler (Cetus) for one cycle of 94°C (5 mins), 55°C (0.5 mins) and 72°C (1 min), followed by 40 cycles of 94°C (1 min), 55°C (0.5 mins) and 72°C (1 min). The final cycle included an extended incubation at 72°C for 5 mins to ensure complete synthesis of all DNA products.

#### **5.2.6. Nested PCR.**

The first amplification step was carried out as described above, except that the number of cycles was reduced to 25. One µl of this PCR product was then subjected to

a further 25 cycles of a second PCR, using one primer common to the first reaction and a second internal or nested primer.

#### **5.2.7. Reverse transcription.**

To amplify RNA, a reverse transcription step was first carried out to produce cDNA which was then amplified by a nested PCR. The PCR reaction mixture was set up as described above, using 1 $\mu$ l of RNA extract, with the addition of 200U of Moloney-murine leukaemia virus 'Superscript' reverse transcriptase (BRL) and 10U RNasin (Promega). The sample was incubated at 42°C for 30 mins to allow cDNA synthesis to occur, then subjected to nested PCR as described above. The initial denaturation step of the first PCR cycle also heat-inactivated the reverse transcriptase enzyme.

#### **5.2.8. Analysis of PCR products.**

Approximately 20 $\mu$ l of each sample was added to 5 $\mu$ l of 5X loading buffer (see Appendix, Table III), loaded on a 2% agarose gel stained with 1 $\mu$ g/ml ethidium bromide and run at 150V for 1.5 h. The DNA bands were visualised and photographed under UV illumination.

#### **5.2.9. Asymmetric PCR for production of single-stranded DNA for sequencing.**

In order to produce a sufficient amount of DNA for direct sequencing, reamplification of the PCR product was carried out using an asymmetric PCR to generate single-stranded DNA.

The primary PCR product was analysed on an agarose gel as described above and the DNA band purified from the gel by electroelution. The ethidium bromide was removed by extraction with an equal volume of butanol three times. The DNA was then ethanol-precipitated and resuspended in 5 $\mu$ l of HPLC-purified water. Five

duplicate reactions were set up, each containing 1 $\mu$ l of this purified MV DNA and subjected to 40 cycles of amplification as described above, except that one primer was diluted 50-fold, to produce an excess of single-stranded DNA. The samples were pooled and concentrated using a Centricon-30 column (Amicon), which also removed excess nucleotides and primers. The DNA was further purified using an Isogene kit (Perkin-Elmer Ltd.).

#### 5.2.10. Sequencing.

Approximately 100ng of single-stranded DNA was sequenced, using the primer which had been diluted in the asymmetric PCR, at a molar ratio of 10:1 (primer:template). Dideoxynucleotide incorporation was carried out using a modified T7 DNA polymerase kit (Sequenase; USB), following manufacturers instructions and incorporating  $\alpha$ -<sup>35</sup>S-dATP (NEN; >1000 Ci/mmol). The sample was heat-denatured and analysed on an 8% polyacrylamide/7M urea gel, which was run at 2000V, soaked in 10% methanol for 20 mins and dried under vacuum at 80°C for 1 hr. The gel was exposed to  $\beta$ -max hyperfilm (Amersham) and developed the following day.



### 5.3. RESULTS.

#### 5.3.1. Detection of measles virus RNA in subacute sclerosing panencephalitis brain tissue.

Cryostat sections of fresh frozen SSPE brain tissue were fixed and duplicate sections probed *in situ* with the M13 probe Z26 which hybridises to the MV NP gene. Following hybridisation, slides were exposed to nuclear emulsion for 2 weeks. Positive hybridisation was indicated by clusters of silver grains over cells in the section (Figure 5.1.). Infected and uninfected vero cells were used as positive and negative probe controls (not shown).

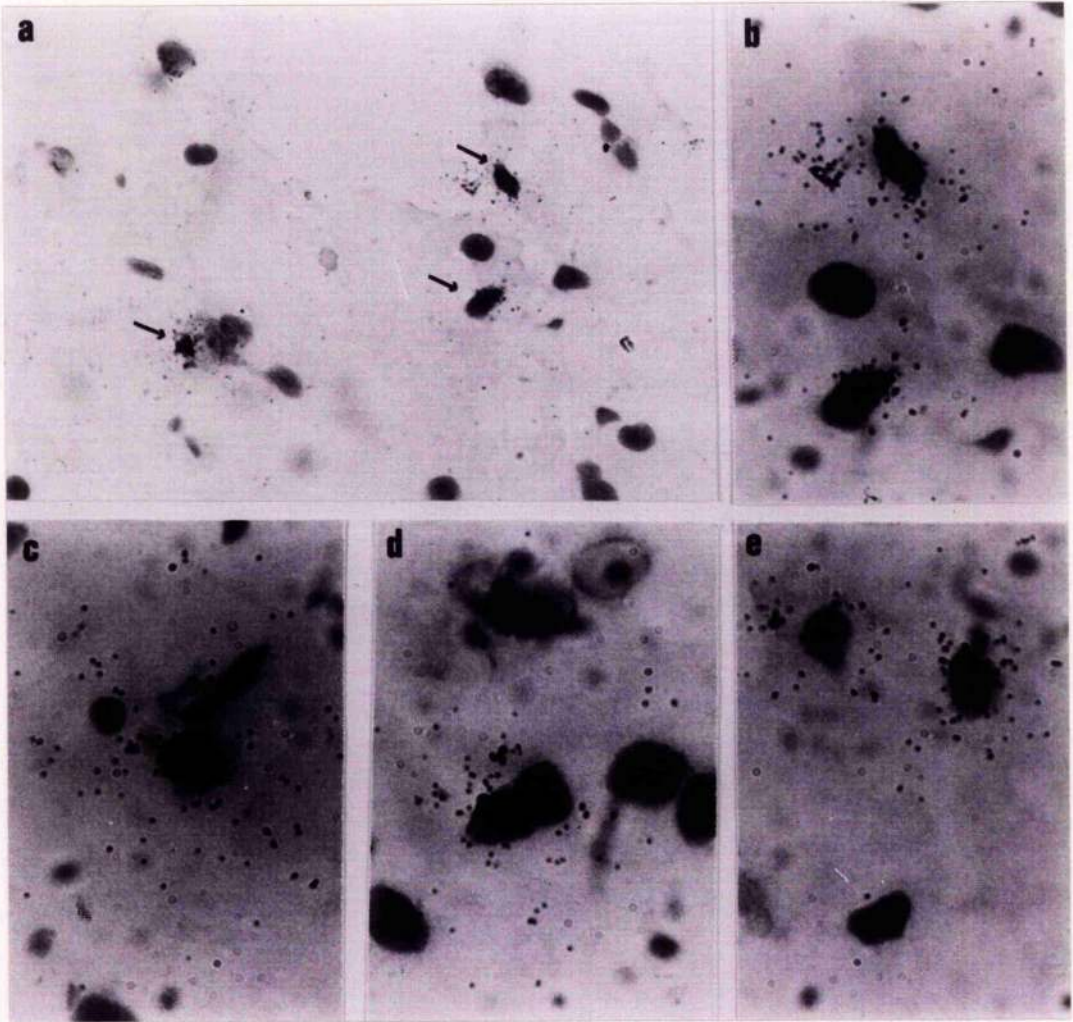
Only a small number of cells in the section showed positive hybridisation and the amount of viral RNA appeared to be relatively low, compared to the amounts found in productively-infected tissue culture cells (see Figure 4.2.), as would be expected from a persistent infection with restricted gene expression. There was no background hybridisation in other areas of the section.

#### 5.3.2. Detection of measles virus RNA in Paget's disease of bone tissue.

##### 5.3.2.1. Detection of MV RNA by *in situ* hybridisation.

Formaldehyde-fixed paraffin-embedded sections obtained from biopsies from five patients with Paget's disease of bone were probed for the presence of various paramyxoviruses by *in situ* hybridisation. Virus-infected and uninfected Vero cells were used as positive and negative controls for each virus probe (not shown) and experiments were carried out in duplicate with varying exposure times of up to 2 months.

Initial experiments were carried out using M13 probes specific for MV (Z26), CDV (112H) and SV5 (4). Negative results were obtained for the SV5 and CDV probes in all five patients. However, low levels of grains were detected over several osteoclasts in one of the sections hybridised with the MV probe (not shown),

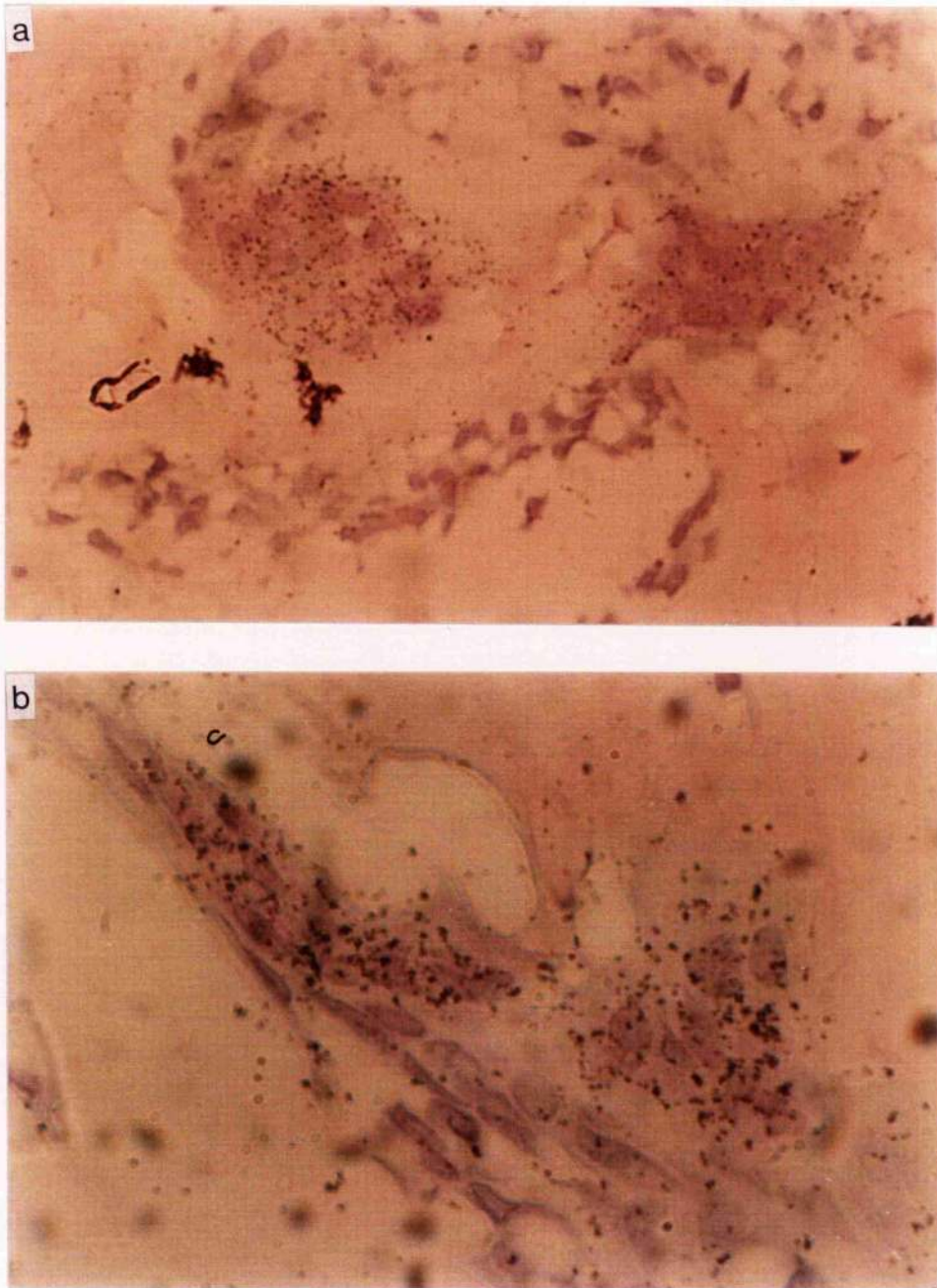


**Figure 5.1. Detection of measles virus RNA in SSPE brain tissue.**

Post-mortem brain tissue was obtained from a patient with subacute sclerosing panencephalitis (SSPE). Frozen sections were cut and fixed with ethanol:acetic acid (3:1) and hybridised with the M13 probe Z26, specific for the MV N gene. Slides were coated with nuclear emulsion and exposed for 2 weeks. Cells showing positive hybridisation are shown in (a)-(e). Two of the cells showing positive hybridisation in (a) are shown at higher magnification in (b).

Stained with haematoxylin and eosin. Magnification (a) x300 (b)-(e) x600.





**Figure 5.2. Detection of measles virus RNA in Paget's bone tissue.**

Formaldehyde-fixed paraffin-embedded sections of Paget's bone tissue were dewaxed and hybridised with the oligonucleotide probe 228924 specific for the MV N gene mRNA. Slides were coated with nuclear emulsion and exposed for 1 month. Positive hybridisation was seen over osteoclasts to varying degrees.

(a) and (b) Strong hybridisation,(above).

(c) Weak hybridisation, (d) No hybridisation, (next page).

Stained with haematoxylin and eosin. Magnification (a) x300 (b)-(d) x600.

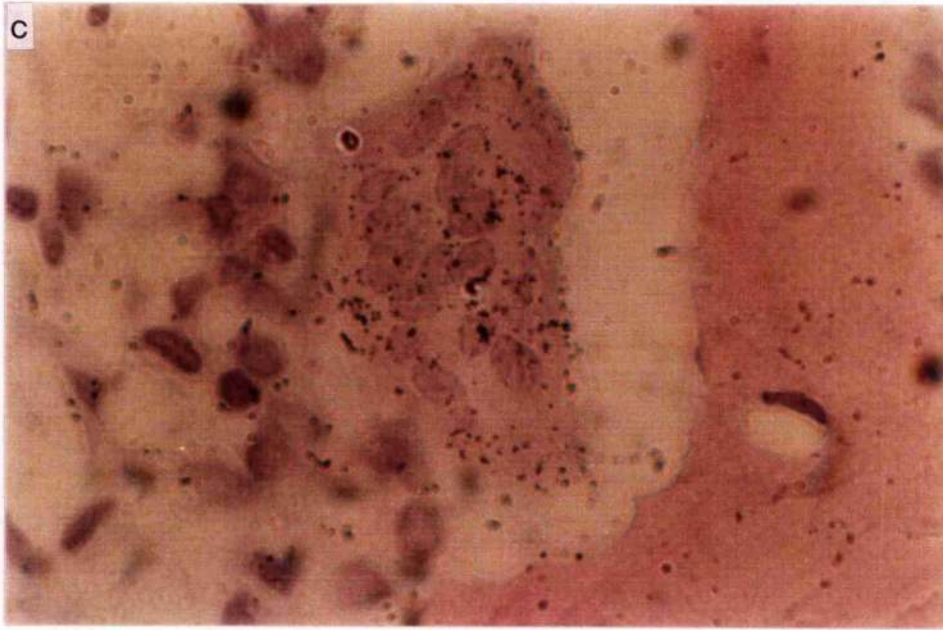


Figure 5.2. (continued from previous page).

although the levels were not strong enough to be classed as a truly positive result, even with exposure times of 2 months. Since a positive control probe for bone tissue was not available, it was not known whether these results were true negative results, or whether problems in penetration were occurring due to the different nature of bone tissue, compared to other tissues previously used.

A smaller probe was therefore used to see whether it could penetrate the bone tissue more effectively. The sections were reprobbed with the MV 33mer oligonucleotide probe (228924) which hybridises to the NP mRNA. Positive hybridisation was observed in this patient when duplicate sections were hybridised with this probe (Figure 5.2.), indicating that penetration of the larger M13 probes was prevented in these sections. The other four patients still gave negative results for MV hybridisation.

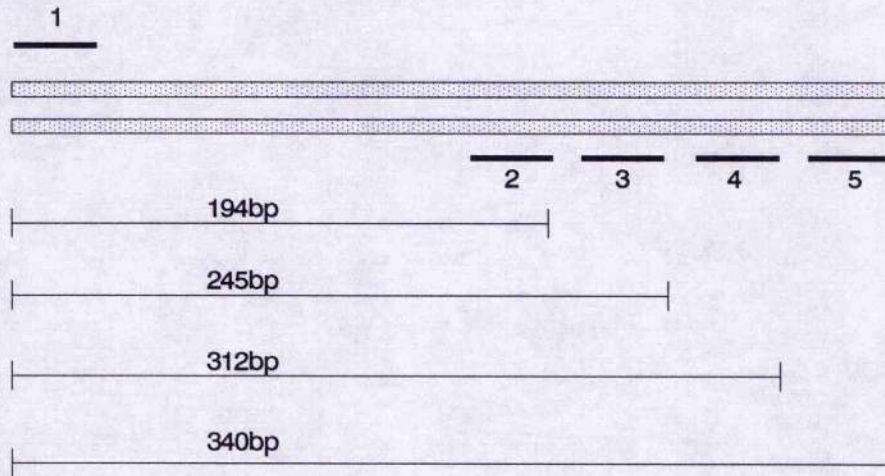
Hybridisation in this patient was detected only over osteoclasts, with varying degrees of hybridisation, as shown in Figure 5.2.(a)-(d). Approximately 30-40% of the osteoclast cells were estimated to show positive hybridisation. Hybridisation was not observed over any other cell type, such as osteocytes, osteoblasts or haematopoietic cells.

#### 5.3.2.2. Amplification of MV RNA by nested reverse transcriptase PCR.

To confirm the presence of MV RNA, a nested reverse transcriptase PCR amplification was carried out on RNA extracted from sections. Five oligonucleotide primers were synthesised specific for the C-terminal end of the N gene of MV which is relatively strongly conserved and shares only low homology with CDV. Each primer shared only 25-30% homology with CDV (Figure 5.3.).

RNA was extracted from five sections of each of the five patients and subjected to nested reverse transcriptase PCR using Primers 1 and 3, followed by Primers 1 and 2. The samples were analysed on a 2% agarose gel stained with ethidium bromide (Figure 5.4.). Control samples were a negative control in which RNA was omitted

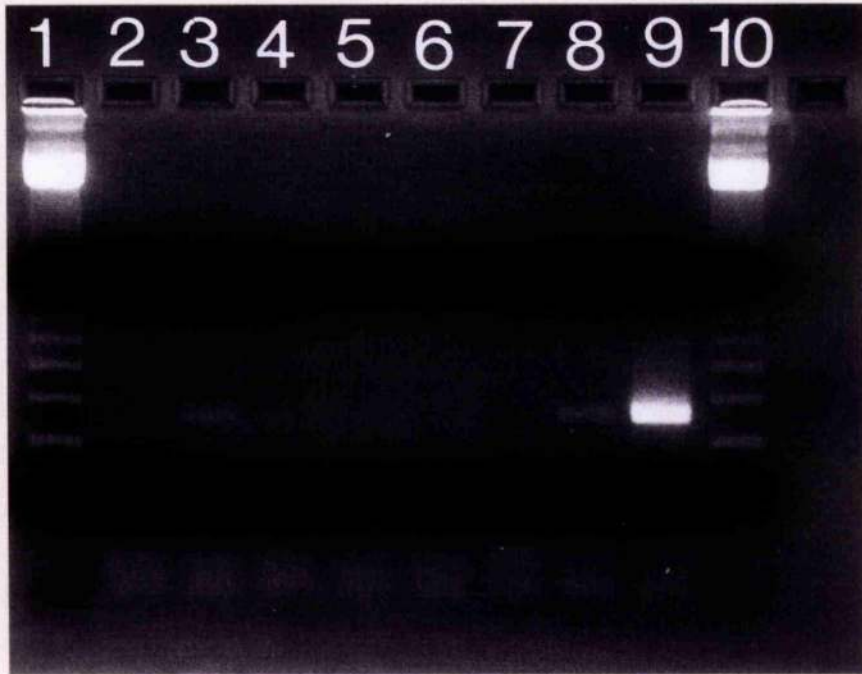




	<b>Nucleotides<sup>a</sup></b>	<b>Sequence.</b>	<b>% homology with CDV.</b>
Primer 1.	1275-1294	5' TGAGAATGAGCTACCGCGAT 3'	30%
Primer 2.	1349-1468	5' TTCGACTGTCCTGCGGATCT 3'	25%
Primer 3.	1500-1519	5' TCTCCGAGATTCTGCCAT 3'	25%
Primer 4.	1567-1586	5' CTCTCGCACCTAGTCTAGAA 3'	25%
Primer 5.	1595-1614	5' GTAGGCGGATGTTGTTCTGG 3'	25%

a. Rozenblatt et al, 1985.

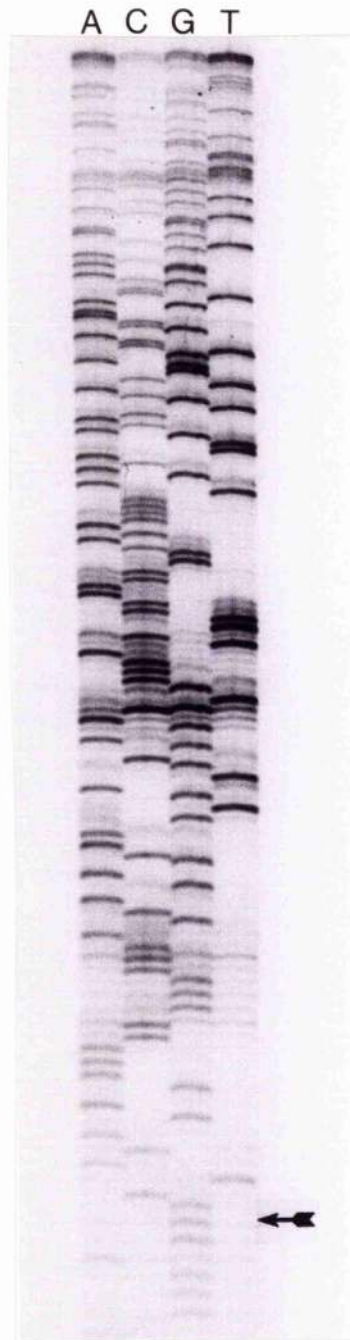
Figure 5.3. Measles virus oligonucleotide primers used for PCR amplification of the MV N gene and direct sequencing.



**Figure 5.4. PCR amplification of MV RNA from Paget's disease of bone tissue.**

RNA was extracted from paraffin-embedded sections of bone and subjected to nested reverse transcriptase polymerase chain reaction amplification of the MV N gene. The PCR product (194bp fragment) was analysed on a 2% agarose gel stained with 1 $\mu$ g/ml ethidium bromide and visualised under UV light. Lanes 1 & 10, 123bp marker ladder; lanes 2-6, RNA extracted from bone of five patients with Paget's disease of bone; lane 7, negative control (no RNA); lane 8, RNA extracted from an SSPE brain; lane 9, cDNA plasmid containing MV N gene. Positive amplification was seen only in one of the five patients (lane 3).





**Figure 5.5. Direct sequence analysis of the amplified MV product.**

DNA was produced by PCR amplification of RNA extracted from a patient with Paget's disease of bone and reamplified by an asymmetric PCR to produce single-stranded DNA for direct sequence analysis. The DNA was purified and approximately 100ng was sequenced using modified T7 DNA polymerase (Sequenase) with incorporation of  $^{35}\text{S}$ -dATP. The reaction was analysed on an 8% polyacrylamide/7M urea gel and exposed to  $\beta$ -max hyperfilm overnight. Approximately 150 nucleotides of sequence were obtained. A single base mismatch was identified compared to the Edmonston strain of MV (arrow).

(lane 7), and two positive controls of RNA extracted from an SSPE brain (lane 8) and a cDNA plasmid containing the MV N gene (lane 9). Of the five patients samples (lanes 2-6), only one produced a positive result with a band of 194bp (lane 3). This correlated with the positive result obtained for this patient by *in situ* hybridisation (Figure 5.2.)

#### 5.3.2.3. Direct sequence analysis of the MV amplified product.

To provide definitive proof that the amplified DNA was MV-specific, single-stranded DNA was produced from an amplified product by an asymmetric PCR and sequenced by direct dideoxynucleotide sequencing. A nested reverse transcriptase PCR was carried out using Primers 1 and 5, followed by Primers 1 and 4 (Figure 5.3.). This product was then purified and subjected to asymmetric PCR, using Primers 1 and 3, with Primer 1 diluted 50-fold to produce an excess of single-stranded DNA. Direct sequencing was carried out using Primer 1 and the result shown in Figure 5.5. Approximately 150 bases could be read and showed >97% homology with the published sequence for the Edmonston strain of MV (Rozenblatt et al,1985), which was also independently sequenced from the cDNA plasmid (not shown). A single base mismatch was identified (Figure 5.5., see arrow; nucleotide 1348). This confirmed that contamination with the cDNA plasmid had not occurred during amplification.

#### 5.4. DISCUSSION.

It is in the study of chronic diseases of viral aetiology that *in situ* hybridisation has become a technique of major importance. A frequent theme in the establishment of persistent infections is a reduction in the levels of virus proteins, which may indeed be a prerequisite for escaping detection by the immune system of the host. Hence immunocytochemical methods are often inadequate and *in situ* hybridisation is at present the only technique which allows sensitive detection together with anatomical resolution. Its sensitivity over other hybridisation techniques comes from its ability to detect virus in highly focal infections, since it avoids the inevitable decrease in sensitivity which results from dilution of the viral nucleic acid in the nucleic acid content of the cells. In addition, the ability to determine the cell types and sites of virus persistence are of particular importance in heterogeneous tissues such as the central nervous system, a frequent site of viral persistence. Although the recently developed gene amplification technique of the polymerase chain reaction provides increased sensitivity over *in situ* hybridisation and will therefore be useful for the detection of putative aetiological agents, it gives no anatomical information about the distribution of virus and the specific cell types involved. The development of an *in situ*-PCR technique for use in infected tissue would be extremely useful for sensitive detection of low levels of viral nucleic acids in such situations.

In these studies, the presence of measles virus RNA was demonstrated in tissue derived from two human chronic diseases, subacute sclerosing panencephalitis (SSPE) and Paget's disease of bone.

MV RNA was detected in two duplicate sections of SSPE brain, indicating that the single-stranded M13 probe used was sensitive enough to detect low levels of viral RNA. Identification of the cell types involved was not possible from these sections, but is most likely to be neurons by analogy with other studies (Haase et al,1985b; Cosby et al,1989). Only a few cells in the section gave positive hybridisation. This



can most probably be explained by the highly focal nature of the disease (Cosby et al,1989). The probe used was specific for viral RNA and therefore could not detect transcription of the N gene. In a study by Haase et al (1985b), the levels of both genomic and mRNA were shown to be restricted and reduced in SSPE cells compared to productively-infected tissue culture cells. In these studies, localisation of the presence of MV within the brain was initially detected by  $^{125}\text{I}$ -exposure to X-ray film, so that focal areas containing MV could be readily identified for more detailed analysis at the cellular level. Fluctuations in the levels of detectable proteins were also observed. Thus, levels of NP appeared to accumulate throughout the course of the disease, being undetectable in early stages, but identifiable at later stages. The M protein was not detectable at either stage, although mRNA was shown to be present at both stages by *in situ* hybridisation, suggesting a specific block in translation. This emphasises the importance of *in situ* hybridisation as a method of detection of virus in persistent infections where restricted gene expression may occur rather than methods of protein detection.

The presence of MV-specific RNA was demonstrated by *in situ* hybridisation in duplicate bone sections from a patient with Paget's disease of bone. Initial problems were encountered which appeared to be due to problems of penetration of the large M13 probes. The conditions used had not been optimised for use with bone tissue, which is different in nature to other tissues previously used. A previous *in situ* hybridisation study on Paget's tissue used smaller nick-translated probes and a higher proteinase K concentration for deproteinisation (Basle et al,1986). A control probe for positive hybridisation in bone tissue (e.g. actin) was not available which could have been used to test this. However, positive hybridisation results were obtained using a smaller oligonucleotide probe (33mer) specific for MV, indicating that penetration problems were the most likely cause of lack of hybridisation with the M13 probe, since the same probe had been successfully used to detect low levels of MV in SSPE tissue. Although this oligonucleotide probe shares some homology

with CDV, hybridisation was carried out under conditions of high stringency, under which it has previously been shown that no cross-reaction occurs (Chapter Two). The presence of MV RNA in this patient was subsequently confirmed by amplification of MV RNA using a sensitive nested reverse transcriptase PCR with MV-specific primers, and by direct sequencing of the PCR product. The use of this technique also failed to detect MV RNA in any of the other four patients, confirming the result obtained by *in situ* hybridisation.

The use of a single-stranded probe for *in situ* hybridisation indicated that transcription of at least the NP gene was occurring. Nucleoprotein mRNA was detected in 30-40% of the osteoclast cells but not in any other cell type. This is in contrast to a previous study in which MV-specific RNA was detected *in situ* in mononucleated cells, in addition to the osteoclasts (Basle et al,1986). These authors reported MV RNA in 80-90% of the osteoclasts and 30-40% of the mononucleated cells, including osteoblasts, osteocytes, fibroblasts, lymphocytes and monocytes. The probe used was a double-stranded nick-translated plasmid specific for the nucleocapsid gene and hence was unable to distinguish between genomic and mRNA. Previous ultrastructural and immunocytochemical studies have shown that paramyxovirus nucleocapsid structure can be demonstrated in the cytoplasm and nuclei of osteoclasts by electron microscopy (Gheradi et al,1980; Rebel et al,1980b; Harvey et al,1982; Howatson & Fornasier,1982) and MV nucleoprotein demonstrated in the cytoplasm and nuclei of osteoclasts (Basle et al,1979; Rebel et al,1980a) but give no evidence of either nucleocapsid structures or viral proteins in any other cell types. However, the hybridisation study by Basle et al (1986) may indicate that MV RNA can persist in cell types other than osteoclasts, but that transcription may be restricted and hence proteins and nucleocapsids are not detectable. Thus, it would be of interest to repeat these studies using complementary single-stranded probes to see whether transcription of MV occurs in a more limited range of cell type than sequestration of the viral genome.

Unfortunately further probing of Paget's sections with oligonucleotide probes specific for other paramyxoviruses, such as CDV and SV5, was not possible because of a lack of further available sections. However, analysis of the presence of the nucleic acids of other paramyxoviruses by *in situ* hybridisation is necessary, considering the numerous reports of the detection of antigens of a variety of paramyxoviruses in Paget's bone tissue. It appears that paramyxoviruses can persist in bone tissue, as previously reported in bone marrow (Goswami et al,1984). However, their presence alone does not necessarily indicate a role in the pathogenesis of Paget's disease of bone. It may be that their presence in osteoclasts is simply a reflection of the long life capacity of these cells and an ability to survive infection by one or more paramyxoviruses. Further studies therefore need to address the question of whether infection with a virus is able to alter the behaviour of a cell and hence result in the disease symptoms seen. Detailed studies of the pathogenesis of Paget's disease of bone have been hampered by the lack of a good animal model. There is one report of the detection of CDV in the osteoclasts of gnotobiotic dogs infected with CDV but no signs of bone deformation were reported (Axthelm & Krakowka,1986). However, further investigation may result in a possible model system for Paget's disease of bone.

*In situ* hybridisation is thus an invaluable technique in the study of chronic persistent infections, and its use in studies of other diseases of proposed viral aetiology, such as autoimmune chronic active hepatitis, systemic lupus erythematosus, chronic glomerulonephritis and multiple sclerosis should aid the resolution of many of the outstanding questions as to the role played by viruses in a range of human diseases.

**CHAPTER SIX.**

**Analysis of the immediate-early gene  
expression of herpesvirus saimiri.**

## 6.1. INTRODUCTION.

Interest has arisen in herpesvirus saimiri because of its close similarity to the human herpesvirus, Epstein-Barr virus, both in disease pattern and at the genomic level. For example, both viruses establish latent infections in the lymphoid cells of their natural hosts and both have an oncogenic potential.

In this chapter, *in situ* hybridisation has been used to examine the expression of the two immediate-early genes of herpesvirus saimiri *in vitro* within individual cells of a productively-infected population. The technique offers the ability to analyse differential gene expression at the mRNA level.

### 6.1.1. Replication of herpesviruses.

Herpesviruses are double-stranded DNA viruses which replicate in the nucleus of infected cells. Following attachment of the virus to cellular receptors, fusion of the viral and cell surface membranes occurs, with release of the capsid into the cytoplasm. The capsid is then transported to the nucleus and the DNA enters the nucleus through the nuclear pores. Transcription and replication occur in the nucleus. Translation of mRNA in the cytoplasm is followed by assembly of the capsid in the nucleus. Replicated DNA is packaged into pre-assembled capsids and the virus matures by budding through the inner nuclear membrane, into which viral glycoproteins have been inserted. The mature virion is then transported to the cell surface and released.

Viral DNA is transcribed by the host cell RNA polymerase, although viral proteins are also involved as co-factors (Costanzo et al,1977). Expression of viral genes can be divided into three main temporal phases, immediate-early (IE), delayed-early (DE) and late (L), which are sequentially transcribed and tightly regulated (Honess & Roizman,1974; 1975). IE genes are expressed first and do not require *de novo* protein synthesis. Immediate-early proteins act as co-factors for



transcriptional activation of DE and L genes, and at least one IE gene product is essential for productive replication in all herpesviruses studied so far. DE genes code mainly for proteins involved in DNA synthesis, such as thymidine kinase, DNA polymerase and DNA binding proteins. DE proteins are also involved in the switch off of IE genes and host protein synthesis, and in the initiation of transcription of L genes. Late proteins comprise the largest group and are mainly structural. For efficient synthesis of L proteins, prior replication of the viral DNA is also required.

A common property of all herpesviruses is the ability to remain latent in their natural host. In this state, expression of the viral genome is repressed such that the genome is maintained within the cell but infectious virus is not produced. During the establishment of latency, normal gene expression is thus repressed at an early stage. Since IE genes play an essential role in switching on the replicative cycle, control of IE gene expression is likely to be an important step in the choice between latency and productive replication.

#### **6.1.2. Herpesvirus saimiri.**

The natural host of herpesvirus saimiri is the squirrel monkey (*Saimiri sciureus*) (Melendez et al, 1968; Falk et al, 1972; 1973). Natural infection occurs within the first years of life and the virus is spread horizontally throughout the population via oral secretions (Falk et al, 1973). Following primary infection, a latent infection of the T lymphocytes is established (Wright et al, 1976). Infectious virus can be recovered from T cells by cocultivation with permissive fibroblastic cells or culture of latently-infected T cells (Falk et al, 1972). The latent infection appears to be maintained during the life of the monkey, indicated by the high success rate of virus isolation from the lymphocytes of seropositive adults (Falk et al, 1972; Deinhardt et al, 1973).

No overt symptoms of disease are observed during infection of the natural host (Ablashi et al, 1976). In contrast, experimental infection of a number of New World

monkey species results in rapidly fatal progressive T-cell lymphomas and leukaemias (Melendez et al,1969; Wolfe et al,1971) which develop regardless of age of the animal or inoculating dose. Neither primates of the Old World species nor humans appear to be susceptible to HVS infection. However, lymphomas can be induced in rabbits in which the pathology shows many similarities to the disease pattern seen in susceptible primates (Daniel et al,1974; Rangan et al,1976) and therefore may provide a more convenient model for studies of virus-host relationships.

#### 6.1.2.1. Comparison of herpesvirus saimiri and Epstein-Barr virus.

Herpesvirus saimiri (HVS) and Epstein-Barr virus (EBV) both belong to the lymphotropic  $\gamma$ -subclass of the herpesviruses. Both have an oncogenic potential and have recently been shown to be closely similar at the genomic level in both gene organisation and sequence homology. The major difference is in their cell tropism for T cells (HVS) or B cells (EBV).

EBV is a natural infection of man, which does not normally result in overt disease symptoms in young children, although it causes infectious mononucleosis in older children and adults. Infection with EBV results in latent infection of the B lymphocytes. It has also been shown to be associated with two human cancers, Burkitts lymphoma, a major childhood cancer in Africa, and nasopharyngeal carcinoma, a common human cancer with high incidence in south-east Asia, Alaska and Greenland. Development of these cancers is therefore also related to additional aetiological and/or genetic factors. Studies of EBV have been hampered by the lack of a truly permissive cell culture system. Although EBV readily infects human and some primate B cell lines *in vitro*, infection results in transformation of the cells and the virus genome remains latent. Infectious virus is produced spontaneously from only a very small percentage of cells (1 in  $10^3$ - $10^6$  infected cells). In contrast, HVS readily undergoes productive infection in a number of cell lines

making *in vitro* studies easier. HVS has also been shown to immortalise T cells from the peripheral blood of marmosets, following *in vitro* infection (Desrosiers et al,1986).

HVS and EBV have recently been found to be closely related, despite major differences in nucleotide composition and in the nature and arrangement of DNA repeat sequences (Gompels et al,1988; Nicholas et al,1988). The EBV genome (172kb) contains mostly coding sequences which have an average G+C content of 60%. These sequences are bounded by direct terminal repeats of 0.5kb. EBV also has a number of internal direct repeats. In contrast, the HVS genome (160kb) is composed of 111kb of coding sequences with a G+C content of 36%, bounded by direct terminal repeats of a 1.44kb sequence with a G+C content of 71% (Bankier et al,1985; Staminger et al,1987). Nevertheless, HVS has been shown to be closely related to EBV. Serological studies have shown similarities between HVS and EBV in both structural and non-structural proteins (Morgan,1977; Blair & Honess,1983; Randall et al,1983) and recent sequencing studies have shown that the overall order and orientation of genes is very closely similar (Gompels et al,1988). Thus, of thirteen open reading frames (ORF) identified throughout the genome of HVS, all were found to be homologous to EBV sequences, whilst only three showed significant sequence homologies to equivalent genes of the  $\alpha$ -herpesvirus, varicella-zoster virus (VZV) (Gompels et al,1988). For example, it has previously been shown that the 160K tegument protein of HVS, located at the far right of the genome is homologous to the equivalent 140K protein of EBV which is found at the far left of the genome (Cameron et al,1987). More recently, the 52kK protein of HVS (see below) and the EB2 protein of EBV, both essential transactivators, have been found to be highly conserved both in sequence and function (Nicholas et al,1988). In addition, an ORF in the HVS genome which is essential for transformation has been mapped near to the junction between coding and repetitive DNA, a location which corresponds to the location of the latent-membrane protein of EBV, which also

possesses transforming activity, although no sequence homology was observed between these two genes (Murthy et al,1989).

EBV and HVS thus share many similar features and studies of gene expression in HVS may have relevance to latency and oncogenicity in EBV. The availability of animal models for HVS infection and the ready establishment of both productive and latent infections *in vitro* should facilitate these studies.

#### 6.1.2.2. Immediate-early gene expression in herpesvirus saimiri.

Studies of the establishment of latency in herpesviruses have concentrated on IE gene expression, since this is likely to be the most important stage of the replication cycle in determining whether a productive or latent infection ensues.

IE genes are transcribed in the absence of *de novo* protein synthesis and can be identified through the use of inhibitors of protein synthesis (e.g. cycloheximide which acts by inhibiting the peptidyl transferase activity during the elongation step of protein synthesis). Infection of cells in the presence of cycloheximide results in the accumulation of IE mRNAs (Honess & Roizman,1974; Randall et al,1984b). This is because IE proteins are not synthesised in the presence of cycloheximide and hence the rest of the lytic cycle, including the switch off of IE gene expression, is not initiated. The inhibitory effects of cycloheximide are readily reversed once it is removed, allowing synthesis of increased amounts of IE protein resulting from the accumulated levels of mRNAs (Honess & Roizman,1974; Randall et al,1984b).

A single major IE protein of HVS has been identified in this way, following high multiplicity infection of Owl monkey kidney (OMK) cells *in vitro* in the presence of cycloheximide (Randall et al,1984b). Removal of cycloheximide from the culture medium was followed by addition of <sup>35</sup>S-methionine to follow protein synthesis and actinomycin D to inhibit further transcription. A protein of apparent molecular weight 52,000 (52K protein) was identified as the only IE protein produced in large amounts (Randall et al,1984b). A number of other minor virus-specific polypeptide

species were also identified in cells infected in the presence of cycloheximide but were synthesised at rates at least 10-fold lower than the 52K protein, and have yet to be further identified. The 52K protein was shown to be phosphorylated and to localise exclusively in the nucleus (Randall et al,1984b).

Studies of the replication of HVS in culture have shown that the replication cycle is prolonged or protracted relative to that of the rapidly cytolytic viruses such as herpes simplex virus (Randall et al,1983; Randall et al,1985), and this has been shown to be related to expression of the 52K protein (Randall et al,1985). Fluorescent studies using monoclonal antibodies against the 52K protein and representative proteins of DE and L genes of HVS have followed protein production at the single-cell level within a population of productively infected cells. Expression of the 52K protein was found to be asynchronous throughout the population, i.e. although infection of all cells occurred simultaneously, not all cells started expressing the 52K protein at the same time. Thus, the percentage of cells expressing the protein increased from 10% to 50-70% over a period of 12-36 hours post-infection (Randall et al,1985). In contrast, expression of DE and L proteins was not significantly delayed following expression of the 52K protein. The protracted time course of replication therefore appears to be a result of the asynchronous expression of the 52K protein within the population.

Asynchrony of 52K protein expression was shown to be reduced but not overcome in experiments in which the multiplicity of infection was increased (Randall et al,1985). This effect was seen at multiplicities greater than those required for infection of 100% of the cell population. Thus, at multiplicities which resulted in 100% of the cells undergoing a productive infection, a further increase in multiplicity resulted in a higher proportion of cells expressing the 52K protein at earlier times post-infection. Asynchrony was not related to the cell cycle, as shown by infection of cells which had been synchronised in culture by the addition of aphidicolin, an inhibitor of cellular DNA synthesis (Randall et al,1985). Double



immunofluorescent labelling experiments were carried out in which the passage of virus to the nucleus was followed using an anti-capsid antibody, and related to expression of the 52K protein in the same cells. A partial correlation was observed, such that accumulation of virus capsid around the nucleus could be correlated with early expression of the 52K protein. However, this correlation was by no means complete, and many cells which showed early accumulation of capsid did not show a correspondingly early expression of the 52K protein.

The 52K protein is produced from a 1.6kb transcript from the EcoRI-I and EcoRI-E fragments of the HVS genome (Nicholas et al,1988). A second IE RNA transcript of 1.3kb, which is also transcribed in the presence of cycloheximide, has recently been identified (Nicholas et al,1990). This gene has been mapped to the Hind III-G fragment of the HVS genome and hence will be referred to as the hinG gene. The HindIII-G fragment of the HVS genome contains two open reading frames, ORF-1 and ORF-2. However, only a probe specific for ORF-1 detects the 1.3kb hinG mRNA. This ORF is predicted to code for a protein of 249 amino acids, with a molecular weight of 28K (Nicholas et al,1990). Attempts to identify this protein by the use of peptide-specific antisera have so far been unsuccessful (Nicholas et al,1990). A minor protein species of apparent molecular weight 30K produced under IE conditions (Randall et al,1984b) is a possible candidate, but the very low level of expression of the protein does not correlate with the high level of mRNA. The role of the 52K and hinG proteins in the replication cycle of HVS have yet to be determined. Both proteins have been shown to act as transactivators of various heterologous promoters in transient expression assays (Nicholas et al,1988; 1990). However, transactivation of HVS DE and L promoters has not been demonstrated (Nicholas et al,1990).

No sequence homology has been identified between the hinG protein and any other herpesvirus protein (Nicholas et al,1990). However, it does show significant homology to a region of the protein encoded by the long ORF in the long terminal

repeat (LTR) of mouse mammary tumour virus (Nicholas et al,1990). The function of this protein is not known so the significance of this finding remains unclear.

In contrast, the 52K gene is highly homologous to the EB2 gene of EBV, both in sequence and function, and more distantly related to the IE 63K gene of herpes simplex virus (HSV) and the IE 68K gene of varicella-zoster virus (VZV) (Nicholas et al,1988). The EB2 protein of EBV has been shown to act as a potent transactivator of heterologous genes, its own promoter and at least one EBV gene, the DR promoter, in transient expression assays (Laux et al,1985; Chevallier-Greco et al,1986; Lieberman et al,1986; Wong & Levine,1986). The 52K protein substitutes efficiently for the EB2 protein in transactivation of the DR promoter, whilst the 63K protein of HSV was a much weaker inducer (Nicholas et al,1988). The strong conservation of sequence between the 52K and EB2 genes therefore also appears to be reflected in their ability to substitute efficiently for each other in functional assays, suggesting a similar mechanism of action. EB2 is switched on by the EB1 protein which plays an essential role in the reactivation of EBV from a latent to a lytic state in culture (Countryman & Millar,1985; Countryman et al,1987). Although several other immediate-early genes have been identified, only the EB1 protein has been shown to be able to induce the lytic cycle on its own (Grogan et al,1987; Rooney et al,1988). EB1 has been shown to bind specifically to sites for the transcription factor, AP1 (Lee et al,1987a,b) which are found upstream of the EB2 gene (Lieberman et al,1986; Wong & Levine,1986). The 52K gene of HVS also has two potential AP1 binding sites (Nicholas et al,1988) and AP1 sites are found upstream of the IE 68K gene of VZV (Davison & Scott,1986) which may indicate a similar mechanism of activation of these three protein homologues. The tumour promoter, TPA (12-O-tetradecanoyl phorbol-13-acetate), has been shown to affect cellular gene expression through factors that bind at AP1 sites (Angel et al,1987) and has been used to enhance reactivation of EBV by EB1 (Farrell et al,1989). However, despite a previous report that TPA reduced the replication cycle time of

HVS (Modrow & Wolf,1983), recent studies failed to show any effect on expression of the 52K protein in TPA-treated infected cultures (Randall et al,1985). Two non-identical palindromic sequences have also been found upstream of both the 52K and EB2 genes (5mer and 11mer in HVS, 6mer and 9mer in EBV) (Nicholas et al,1988), although the significance of these is as yet unknown.

Studies of the expression of the 52K and hinG mRNAs by Northern blot analysis have indicated that the hinG mRNA accumulates earlier in infection than the 52K mRNA (Nicholas et al,1990). However, studies of the hinG protein have been hampered by the lack of antibodies to the protein, preventing immunocytochemical analysis of its expression at the single cell level. In this chapter, the relative expression of the hinG gene has therefore been compared to the 52K gene. Oligonucleotide probes specific for the 52K and hinG mRNAs were designed and used to analyse the regulation of expression of each gene within a population of productively-infected cells, by hybridisation to mRNA.

## 6.2. MATERIALS AND METHODS.

### 6.2.1. Infection of monolayers.

Monolayers of Vero cells were grown on multiwell slides as described in Section 3.2.1.3. Cells were infected with herpesvirus saimiri (HVS) strain 11'O' at a multiplicity of 100 p.f.u./cell, diluted in growth medium unsupplemented with calf serum. Virus was adsorbed for 1.5 h at 37°C. The slides were then flooded with medium supplemented with 2% calf serum, with or without the addition of cycloheximide to a final concentration of 50µg/ml and incubated at 37°C. At various times post-infection (p.i.), cells were fixed with either ethanol:acetic acid (3:1) for *in situ* hybridisation studies or with 5% formaldehyde, 2% sucrose, for immunocytochemical studies.

### 6.2.2. *In situ* hybridisation.

Oligonucleotide probes specific for the 52K and hinG mRNAs were labelled with <sup>35</sup>S-dCTP by primer extension, as described in Section 2.2.2.6.

*In situ* hybridisation was carried out as described in Chapter Three. Cells were pretreated with 0.2M HCl, 1µg/ml proteinase K and dehydrated in graded alcohols. Both probes were used at a concentration of 1ng/ml and hybridisation was at 37°C for 24 h. Post-hybridisation washing steps included a high stringency wash at 60°C in 2X SSC. Detection of hybridisation was initially by exposure to X-ray film for up to 1 month, followed by exposure to nuclear emulsion for 2 months. Following development of the emulsion, the cells were stained with 1% toluidine blue.

### 6.2.3. Immunoperoxidase detection of the IE 52K protein.

Cells were fixed, permeabilised and a monoclonal antibody against the 52K protein adsorbed as described in Section 4.2.4. Binding of antibody was detected by the addition of 10µl per well of horseradish peroxidase-linked goat anti-mouse polyclonal antibody (preadsorbed against Vero cells). The slides were incubated at

room temperature for 1 h, washed 4-5 times with PBS'A' and once with dH<sub>2</sub>O.

Binding of antibody was visualised by addition of the chromogenic substrate diaminobenzidine (DAB). The DAB solution was freshly prepared by dissolving approximately 10mg in 20mls 0.05M Tris-HCl pH 7.4, followed by filtration. Hydrogen peroxide (30% stock) was added to a final concentration of 0.06%. The solution was added to the slides and incubated at room temperature for up to 30 minutes, until a colour change was visible. The slides were then washed three times in Tris buffer and thoroughly in dH<sub>2</sub>O. The presence of the 52K protein was indicated by the deposition of an insoluble brown precipitate.



### 6.3. RESULTS.

#### 6.3.1. Labelling of oligonucleotide probes.

Oligonucleotide probe sequences specific for the 52K and hinG genes were selected from published sequence data (Figure 6.1.; Table 6.1.). Probes were labelled with  $^{35}\text{S}$ -dCTP by primer-extension reactions and template oligonucleotide sequences were synthesised so that the resulting probe was specific for mRNA. The resulting specific activities were  $9.2 \times 10^8$  cpm/ $\mu\text{g}$  (52K) and  $1.5 \times 10^9$  cpm/ $\mu\text{g}$  (hinG) (Table 6.1.).

#### 6.3.2. Asynchronous expression of the IE 52K gene.

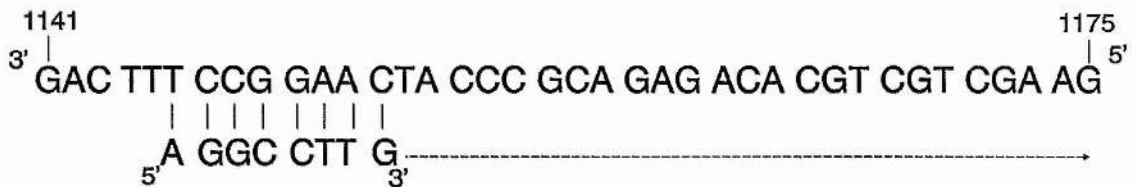
Expression of the 52K gene was analysed by detection of the protein and mRNA. Monolayers of Vero cells were infected with a high m.o.i. of HVS and incubated at 37°C. At 6, 12 and 18 hours p.i., the cells were fixed. The presence of the IE 52K protein was detected by an indirect immunoperoxidase reaction using a monoclonal antibody specific for this protein (Randall et al, 1983), and the 52K mRNA was detected by *in situ* hybridisation.

In cells in which the 52K protein was detected, a brown precipitate was formed in the nucleus, as shown in Figure 6.2. The number of positive cells at each time post-infection (p.i.) was counted under low power magnification fields from 8 wells of 2 duplicate slides and the results shown in Table 6.2.

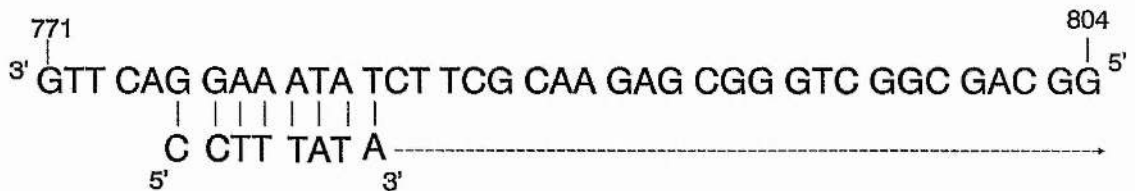
Asynchronous expression of the 52K protein was seen to occur. Thus, although all cells were infected with virus, as indicated by detection of the 52K protein in >99% of cells by 18 hours p.i. (Figure 6.2.), not all cells started to express the protein simultaneously. Thus, the number of positive cells increased from 29% at 6 hours p.i. to 54% at 12 hours p.i. By 18 hours p.i., a cytopathic effect shown by rounding up of the cells was also evident.

*In situ* hybridisation was carried out using an oligonucleotide probe specific for

(a) 52K.



(b) hinG.



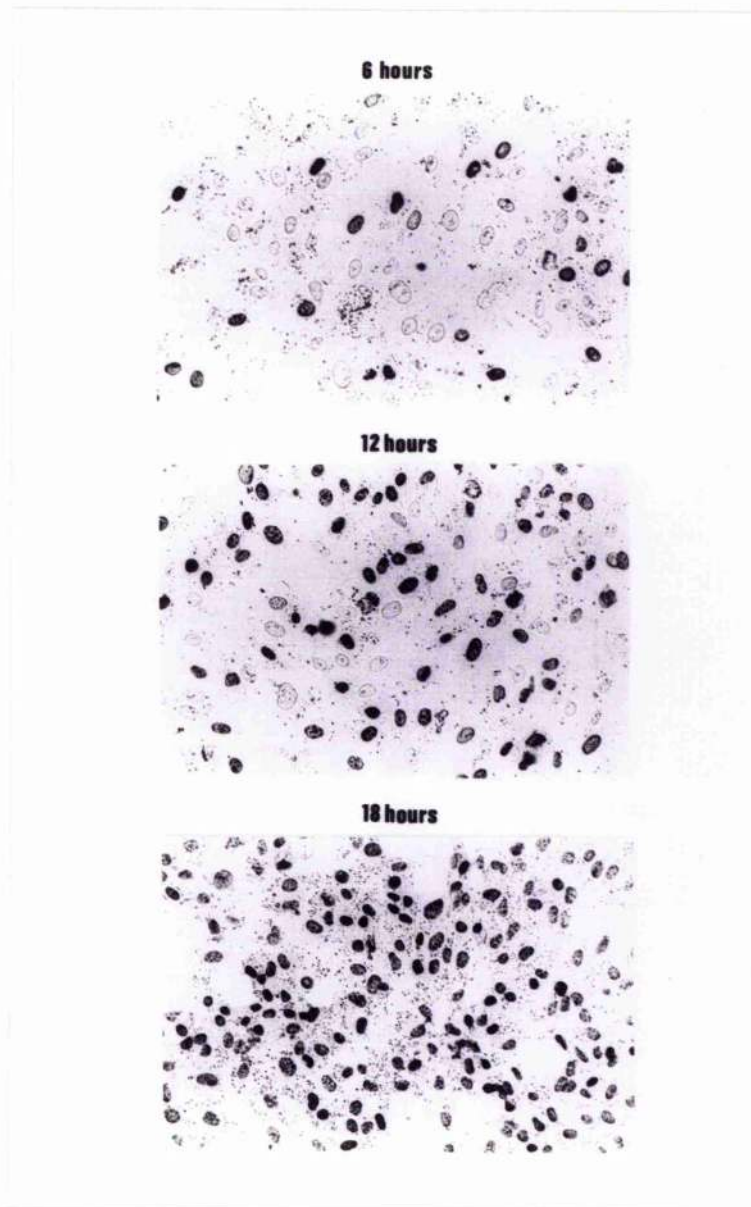
**Figure 6.1. Sequences of the herpesvirus saimiri oligonucleotide probe templates and primers.**

The sequences of the templates and primers were selected from published sequence data (Table 6.1.) and were chosen to have a high G+C content.

Probes were labelled by primer extension, as shown above.

Table 6.1. Details of the herpesvirus saimiri oligonucleotide probes.

Probe	Size	Sense	Sp.Act. (dpm/ug)	% G + C	Nucleotides	Reference
52K	33mer	v	$9.2 \times 10^8$	61	1141-1175	Nicholas et al, 1988
hinG	33mer	v	$1.5 \times 10^9$	61	771-804	Nicholas et al, 1990



**Figure 6.2. Asynchronous expression of the IE 52K protein.**

Monolayers of Vero cells were infected with a high m.o.i. (100p.f.u./cell) of HVS. Cells were fixed at 6, 12 and 18 hours p.i. with 5% formaldehyde, 2% sucrose. The presence of the IE 52K protein was detected by addition of a mouse anti-52K monoclonal antibody, followed by horseradish peroxidase-linked goat anti-mouse polyclonal antibody. Addition of the chromogenic substrate diaminobenzidine (DAB) resulted in deposition of an insoluble brown precipitate indicating the presence of the 52K protein.

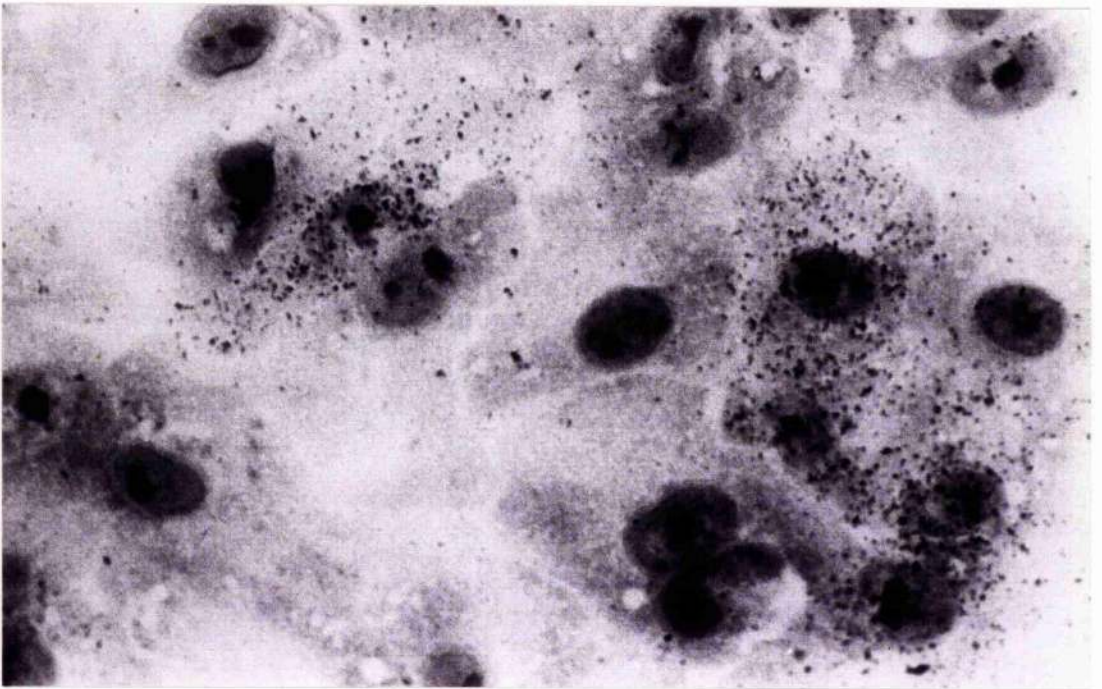
Magnification x150.

Table 6.2. Estimation of the % of cells expressing the IE 52K protein by immunoperoxidase detection.

Time p.i. (hours)	Number of cells counted <sup>a</sup>	Number of positive cells	% positive cells
6	634	184	29
12	546	295	54
18	317	315	>99

a. Counted from low power magnification fields from 8 wells of 2 duplicate slides.





**Figure 6.3. Asynchronous expression of the 52K mRNA.**

Monolayers of Vero cells were infected with a high m.o.i. of HVS in the presence of cycloheximide and fixed at 12 h p.i. with ethanol:acetic acid. *In situ* hybridisation was carried out using an oligonucleotide probe specific for the 52K mRNA. Slides were exposed to nuclear emulsion for 1 month.

Stained with 1% toluidine blue. Magnification x300.

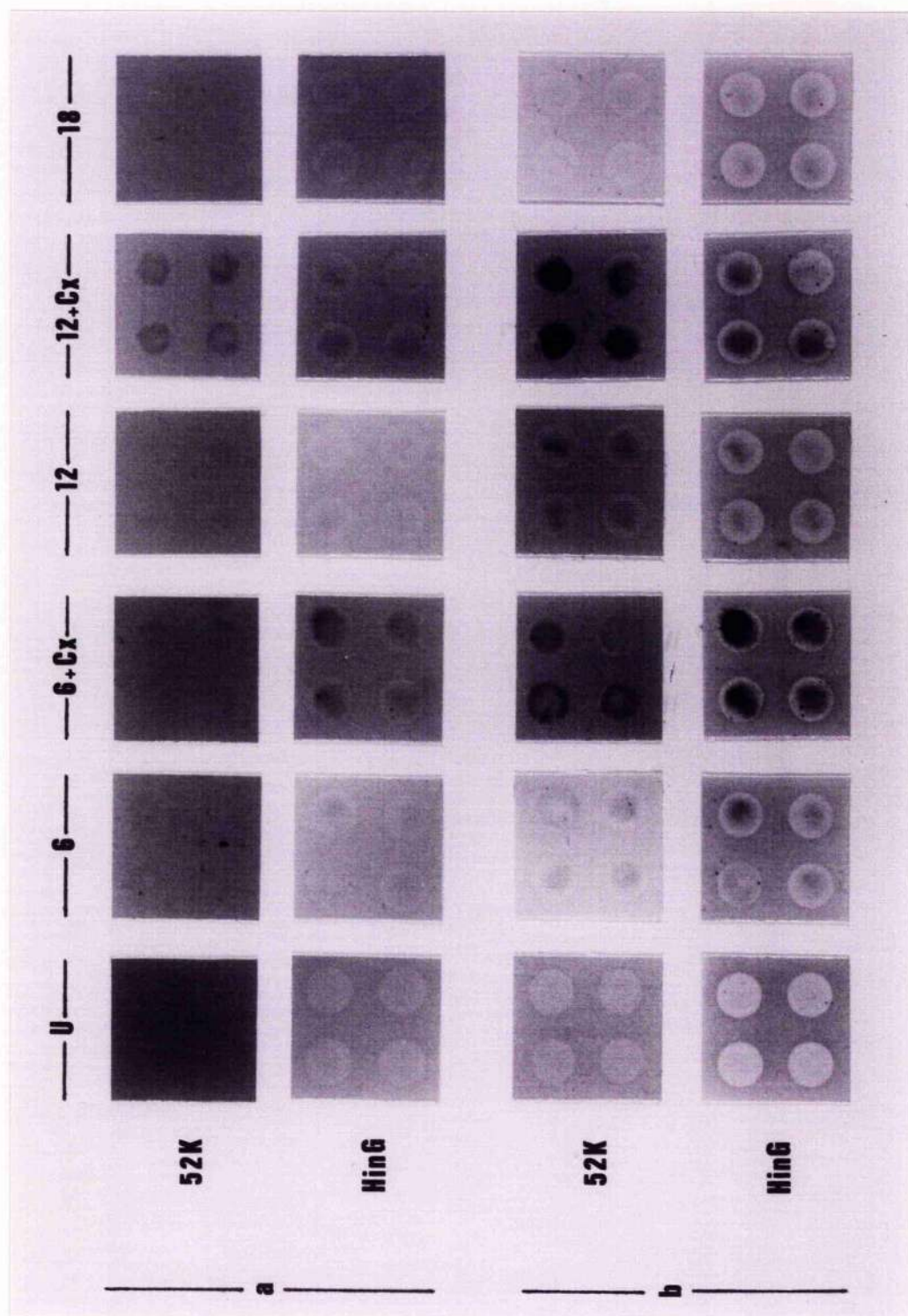
the 52K mRNA, on cells infected in the presence of cycloheximide and fixed at 12 h p.i. Following exposure to nuclear emulsion and development, expression of the 52K mRNA was indicated by the presence of silver grains. Expression of the 52K mRNA was also clearly asynchronous, as shown in Figure 6.3.

### 6.3.3. Differential expression of the 52K and hinG mRNAs.

Monolayers of Vero cells were infected with a high m.o.i. of HVS, in the presence or absence of cycloheximide. At 6, 12 and 18 hours p.i., the cells were fixed and probed *in situ* for the presence of the 52K and hinG mRNAs.

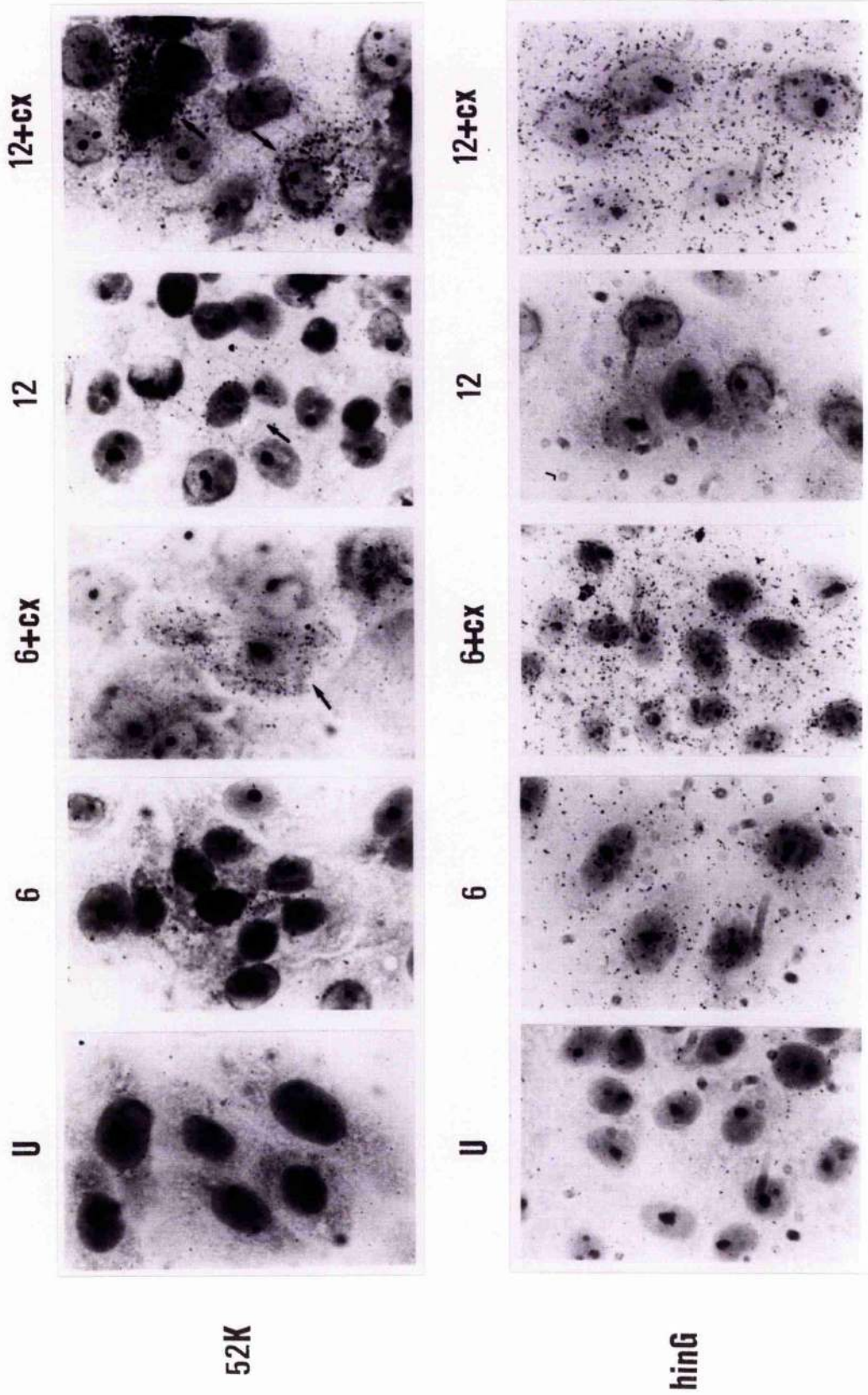
Following hybridisation, the slides were initially exposed to X-ray film for up to one month to assess overall hybridisation levels. Under normal conditions, i.e. no cycloheximide, expression of both the 52K and hinG mRNAs was relatively low (Figure 6.4.). However, in the presence of cycloheximide, expression of both genes was greatly enhanced allowing comparisons to be made (Figure 6.4.). Differences in the expression of the two genes in the presence of cycloheximide were apparent. Expression of the 52K gene appeared to increase from 6 to 12 hours p.i., whereas expression of the hinG gene was maximal at 6 hours p.i.

Slides were then coated with nuclear emulsion and exposed at 4°C for up to 2 months. The results of this analysis at the single cell level are shown in Figure 6.5. Hybridisation was weak in cells infected in the absence of cycloheximide but in cells in which IE gene expression had been enhanced by the presence of cycloheximide, strong hybridisation signals were detected. Expression of the 52K gene was asynchronous in agreement with the immunoperoxidase analysis of the expression of the 52K protein. This asynchrony was shown by differences in the levels of grains over neighbouring cells in the monolayer. The number of cells with strong expression of the gene increased from 6 to 12 hours p.i. in agreement with expression of the 52K protein, shown in Figure 6.5. By 12 hours, the number of



**Figure 6.4.** Differential expression of the 52K and hinG IE mRNAs following exposure to X-ray film. Monolayers of Vero cells were infected with a high m.o.i. (100 p.f.u./cell) of HVS, in the presence or absence of cycloheximide (cx). Cells were fixed at 6, 12 and 18 h p.i. with ethanol:acetic acid (3:1). *In situ* hybridisation was carried out using oligonucleotide probes specific for the 52K and hinG mRNAs. Slides were exposed to X-ray film for (a) 1 week, (b) 1 month.





**Figure 6.5. Differential expression of the 52K and hinG IE mRNAs at the single cell level.**

Monolayers of Vero cells were infected with a high m.o.i. (100 p.f.u./cell) of HVS, in the presence or absence of cycloheximide (cx). Cells were fixed at 6, 12 and 18 h p.i. with ethanol:acetic acid (3:1). *In situ* hybridisation was carried out using oligonucleotide probes specific for the 52K and hinG mRNAs.

Slides were exposed to nuclear emulsion for 2 months  
Stained with 1% toluidine blue Magnification x300.

cells with high levels of expression had increased, although expression was still clearly asynchronous (Figure 6.5.). In contrast, the *hinG* gene appeared to be expressed synchronously throughout the population i.e. all cells showed comparable levels of hybridisation (Figure 6.5.).



#### 6.4. DISCUSSION.

*In situ* hybridisation was used to study expression of the two IE genes of HVS within a population of productively infected cells. Since antibodies were not available against the hinG protein, immunocytochemical analysis of its expression was not possible. Expression was therefore studied at the level of transcription. *In situ* hybridisation is the only method which offers the ability to analyse differential gene expression within a population of cells at the mRNA level, since methods such as Northern blots only give results averaged throughout the cell population.

Comparison of overall hybridisation levels after exposure to X-ray film, indicated apparent differences in the expression of the two genes at various times post-infection. Comparisons were most readily made between cells infected in the presence of cycloheximide, in which levels of IE mRNA were greatly enhanced. Thus, expression of the 52K gene increased from 6 to 12 hours post-infection, whereas the hinG gene appeared to be expressed maximally by 6 hours. This is in agreement with studies by Northern blot analysis in which it has been shown that the hinG mRNA is the predominant stable RNA (Nicholas et al, 1990).

Analysis at the single-cell level also revealed differences in regulation of expression of the two genes. Whereas the level of 52K gene expression was clearly asynchronous, the hinG gene appeared to be expressed synchronously throughout the population.

It has been shown that the 52K protein is expressed asynchronously within a population of productively-infected cells (Figure 6.2.; Randall et al, 1985). Expression of the 52K protein has been shown to precede expression of the DE proteins (Randall et al, 1985) and therefore appears to be an essential step in the productive replication cycle of HVS. It was previously suggested by Randall et al (1985) that the asynchronous expression of the 52K protein was caused mainly as a result of asynchrony of transfer of virus from the cell surface to the nucleus where gene expression can commence, and a partial correlation was demonstrated between

perinuclear accumulation of parental capsid and early expression of the 52K gene. However, the early and synchronous expression of the *hinG* gene which has been demonstrated here indicates a specific control mechanism for 52K gene expression which differs from that of the *hinG* gene in requiring or being inhibited by additional factors. It is possible that regulation of expression of the 52K gene could play a role in the establishment of latency by this virus in its natural host. However, there is as yet no conclusive evidence that the 52K protein is an essential transactivator. Co-transfection experiments with expression vectors containing the 52K gene have been shown to increase chloramphenicol acetyl transferase (CAT) activity from heterologous promoter-CAT constructs in transient expression assays (Nicholas et al,1988) but no activity has been detected using HVS DE and L promoter-CAT constructs as targets for the 52K protein (Nicholas et al,1990).

Differential regulation of the 52K and *hinG* genes has also been indicated by experiments in which the relative levels of mRNA were compared at different multiplicities of infection (m.o.i.). The level of *hinG* mRNA increased 50-100-fold when the m.o.i. was increased from 5 to 25 p.f.u./cell, in the presence of cycloheximide (Nicholas et al,1990). In contrast, the 52K mRNA was undetectable at the lower m.o.i., even with prolonged exposure times, despite being readily detectable at a m.o.i. of 25 p.f.u./cell, indicating that expression of the two genes is differentially affected by the m.o.i. used.

The exact roles of the 52K and *hinG* proteins in the productive replication cycle of HVS therefore remain to be elucidated. However, in contrast to the failure to demonstrate transactivation of HVS DE and L promoters by the 52K and *hinG* proteins, it has been shown that a protein encoded within the EcoRI-D fragment of the genome can transactivate the promoters of the DE 110K DNA-binding protein and the late thymidylate synthase genes (Nicholas et al,1990). Comparisons of the genome structures of HVS and EBV have shown that this fragment is co-linear with the region of the EBV genome containing the BZLF1- (EB1) and BRLF1-encoded

transactivators. Stable mRNA from this region was not detected under IE conditions (Nicholas et al,1990), although it is possible that low levels of protein could be produced from an unstable mRNA which may be undetectable. However, it is also possible that either the 52K or hinG proteins could be involved in the expression of this transactivator.

Regulation of early gene transcription in HVS is obviously complex and requires further investigation. The importance of the present result lies in the demonstration that in a population of infected cells, all of which contain copies of the HVS genome which are active in the transcription of the hinG gene, there is asynchrony in the transcription of the 52K gene. Thus, in addition to cell-to-cell variation in entry and intracellular processing of virus particles which provides one component of asynchrony (Randall et al,1985), it is now clear that the transcription of the 52K gene requires or is prevented by factors which do not affect the transcription of the hinG gene. Thus, there is differential regulation of the expression of these two IE genes. Previous studies have shown that expression of the 52K protein is followed by entry into the lytic cycle of DE and L gene expression (Randall et al,1984b; 1985). The nature of the transcription factors which differentiate between the hinG and 52K IE genes of HVS therefore seem likely to play a crucial role in determining the entry of this virus into the lytic cycle of virus gene expression.

**CHAPTER SEVEN.**

**General discussion and future developments.**

## 7.1. SUMMARY OF RESULTS.

The overall aim of the thesis was to demonstrate the use of *in situ* hybridisation in the detection of viral RNA, by applying the technique to several areas of interest. Thus, hybridisation conditions were optimised for the detection of paramyxovirus RNA using single-stranded DNA and RNA probes. The use of single-stranded probes allowed differential detection of messenger and genomic RNA. DNA probes were found to be superior to RNA probes and were therefore used in subsequent studies.

A mouse model system was developed to examine the host factors involved in protection against virus and clearance of virus from the lungs. It was shown that a reduction in the levels of viral RNA and proteins occurred in the lungs of mice which had been immunised with either internal or external viral proteins, due to the induction of virus-specific cytotoxic T cells which enhanced clearance of virus. Infection of immunodeficient mice was found to result in a persistent infection, in which viral RNA and protein could be detected at prolonged times post-infection, although there was little production of infectious virus. The establishment of a persistent infection allowed a more detailed analysis of the specific cell types involved in virus clearance by transfer of selected cell populations to infected irradiated mice.

The ability to detect measles virus RNA in two chronic human diseases was investigated, in order to demonstrate the potential use of *in situ* hybridisation for detection of putative aetiological agents. Measles virus RNA was detected in brain sections from an SSPE patient, and in osteoclasts in bone sections from a patient with Paget's disease of bone. The ability to detect low levels of viral nucleic acids in such situations, where gene expression is often highly restricted, will be a valuable technique for assessing the role played by viruses in a range of human diseases.

The ability of *in situ* hybridisation to detect differential gene expression within a population of cells was utilised to examine regulation of the expression of the two IE genes of herpesvirus saimiri, by analysis of mRNA expression. Thus, expression



of the *hinG* gene was shown to be synchronous throughout a population of productively-infected cells, in contrast to the 52K gene which appeared to be specifically regulated resulting in an asynchronous pattern of expression. This may have relevance to the mechanisms of establishment of latency in this virus. Further studies of this kind should throw light on possible mechanisms of latency in other herpesviruses, where control of IE gene expression is also likely to play an essential role.

## 7.2. FURTHER DEVELOPMENT OF *IN SITU* HYBRIDISATION TECHNOLOGY.

The use of highly specific probes allows detection of more than one nucleic acid in a single cell, the technique being limited by the availability of different detection systems. A method for double labelling of two nucleic acids in the same cell using radioactively-labelled probes has been described by Haase et al (1985c) in which colour photographic development was used to distinguish between two probes labelled with  $^3\text{H}$  and  $^{35}\text{S}$ . The cells were coated with a layer of emulsion, exposed and developed, and the silver grains reacted with a dye coupler to form a magenta colour. This layer of emulsion was then covered with a thin clear film and coated with a second layer of emulsion. Following exposure and development, the silver grains in the second layer were converted to a different colour. Since only the  $^{35}\text{S}$  particles were able to penetrate the plastic film, hybridisation of the two probes could be distinguished. However, this method is lengthy and complicated, and double-labelling of nucleic acids may be more readily carried out using non-radioactive labels with different detection systems (Hopman et al, 1986; Mullink et al, 1989) or by combining a radioactive and non-radioactively labelled probe (Singer et al, 1987).

Double labelling of nucleic acids by hybridisation and of proteins by immunocytochemistry is readily achieved, using both radioactively and non-radioactively labelled probes. Immunocytochemical detection is generally carried out first using substrates which can withstand the subsequent treatment

required for hybridisation. This has been used, for example, to detect viral genes and their products in the same cell (Blum et al,1984; Brahic et al,1984; Haase et al,1984b; Stowring et al,1985) and to identify specific cells which harbour virus (Stowring et al,1985).

The use of radioactive probes has allowed quantitative studies of nucleic acid levels to be determined by *in situ* hybridisation, since the number of grains in the emulsion can be related to the specific activity of the label used. Quantification requires calibration with known standards in order to relate the number of silver grains over a cell to a known amount of radioactivity. With nuclear emulsions, it must be ensured that variations in the grain density are not simply a result of an uneven emulsion layer and thus isotopes of lower energy are generally used for quantitative studies, such as  $^3\text{H}$  and  $^{125}\text{I}$  (Szabo et al,1977). The technique is, however, problematic and must be strictly controlled. Given the large number of interacting variables which can affect hybridisation and the difficulty of assessing the efficiency of hybridisation, absolute measurements seem likely to be inaccurate.

The results obtained in this thesis were used purely for qualitative analysis of the relative levels of hybridisation under different conditions and no attempt was made to quantitate the number of RNA molecules per cell. However, the results obtained did not appear to approach the level of sensitivity reported by others such as Brahic and Haase (1978), since long exposure times were often required to obtain reasonable grain numbers, despite the use of  $^{35}\text{S}$  rather than  $^3\text{H}$  or  $^{125}\text{I}$ .

### 7.3. FUTURE DIRECTIONS.

With advances in non-radioactively labelled probes for hybridisation studies, and increases in sensitivity, the use of *in situ* hybridisation for diagnosis of viral infections has become a realistic possibility. However, despite development of the technology and examples of its application (Brigati et al,1983; Unger et al,1986; Burns et al,1986; Lewis et al,1987), its use has generally been limited because of

the technical problems of handling large numbers of samples. Development of automated techniques (Unger & Brigati, 1989) should result in more widespread use of such methods, which will be of particular importance for viruses which are not readily cultured such as rotaviruses and papillomaviruses.

The recent development of the polymerase chain reaction (PCR) (Saiki et al, 1985; 1988) is likely to become an important molecular tool in the field of virology for detection of putative aetiological agents and provides increased sensitivity over *in situ* hybridisation. However, it gives no anatomical information and therefore cannot substitute for *in situ* hybridisation in studies of pathogenesis. The development of an *in situ*-PCR technology would be an invaluable technique for rapid and highly sensitive detection of virus. As yet, there is only one report of successful *in situ*-PCR, carried out in cells in suspension, which were then cytospun onto slides and the amplified signal detected by normal *in situ* hybridisation (Bagasra, 1990). Problems with a direct *in situ*-PCR are likely to result from reduced morphological detail, caused by the high temperatures required for PCR, and by the problem of diffusion of the product following amplification.

#### 7.4. CONCLUSIONS.

At present, *in situ* hybridisation remains the only technique which gives sensitive detection of nucleic acids together with highly specific anatomical resolution. As such, it has found particular application in the field of virology, since viral infections are generally highly focal. The ability to detect viral nucleic acids and identify the specific cell types involved in infection is invaluable for studies of pathogenesis, in particular for persistent viral infections, where gene expression may be highly restricted.

The technique of *in situ* hybridisation, in conjunction with other molecular techniques, thus offers new opportunities for a fuller understanding of the molecular mechanisms of viral pathogenesis.

**APPENDIX.**

## I. Purification of nucleic acid samples.

### (a) Extraction with phenol and chloroform.

Contaminating proteins were removed from nucleic acid samples by extracting once with phenol, once with phenol:chloroform:isoamylalcohol (25:24:1) and once with chloroform. Solutions were saturated with 100mM Tris-HCl pH 8.0 and stored under buffer at 4°C. An equal volume of each solution was added to the nucleic acid solution, mixed vigorously and centrifuged for 5 mins at 12,500rpm to separate the two layers. The upper (aqueous) layer containing the DNA was removed and rest discarded.

### (b) Extraction with ether.

Ether was used to remove any remaining traces of phenol or chloroform. An equal volume of ether was added to the nucleic acid solution, mixed vigorously and centrifuged briefly. The upper ether layer was discarded and any remaining ether was evaporated by heating briefly.

### (c) Ethanol precipitation.

DNA or RNA were precipitated from aqueous solution by the addition of 0.1 volumes 3M sodium acetate pH 5.2 and 2.5 vols ethanol. Samples were incubated at -20°C for at least 1 h or in a dry ice-ethanol bath for 10 mins, then centrifuged at 12,500rpm for 10 mins. The pellet was gently washed in 70% ethanol and respun for 5 minutes. The supernatant was removed and the pellet evaporated to dryness under vacuum. The DNA or RNA was resuspended in water or an appropriate buffer.

### (d) Spin-column purification.

This was used for rapid separation of labelled DNA or RNA from unincorporated nucleotides. A 1 ml disposable syringe was plugged with a sterile glass wool and filled with Sephadex G-50 equilibrated with an appropriate buffer. This was placed in a



centrifuge tube and centrifuged at 1,500rpm for 3 mins. The column was repacked and respun until the final bed volume was approximately 0.9mls. The column was equilibrated with 200 $\mu$ l buffer and the sample in a volume of 200 $\mu$ l was then applied to the column and spun exactly as above. The DNA was recovered in a Eppendorf tube placed in the bottom of the centrifuge tube, whilst the unincorporated nucleotides were retained in the column.

## II. Spectrophotometric quantitation of nucleic acids.

The absorbance reading of a nucleic acid sample was measured on a Perkin Elmer spectrophotometer at a wavelength of 260nm. The concentration was calculated from the equations:

$$1 \text{ O.D.} = 50\mu\text{g/ml for double-stranded DNA}$$

and  $1 \text{ O.D.} = 40\mu\text{g/ml for single-stranded DNA, RNA and oligonucleotides.}$

The ratio of  $\text{O.D.}_{260\text{nm}}/\text{O.D.}_{280\text{nm}}$  allowed an estimation of the purity of the sample. For a pure sample this ratio should be 1.8 for DNA and 2.0 for RNA. Contamination with proteins or phenol results in a significantly lower ratio.

## III. Recrystallisation of formamide.

Commercially available formamide was purified from ionic impurities by recrystallisation at 0-4°C. A volume of formamide was stirred in a beaker immersed in an ice-bath at 4°C. When 40-50% of the volume had crystallised, the bulk of the supernatant was discarded and the crystals spun briefly through a syringe fitted with a filter paper plug to remove remaining liquid. The crystals were stored at -70°C and melted when required. For ultrapure formamide, this procedure was repeated.

## IV. Growth of bacteria.

The *E.coli* strain JM101 (supE thi (lac-proAB) F' [tra D36 pro A<sup>+</sup> pro B<sup>+</sup> lac I<sup>q</sup> lac Z M15]) was used for preparation of M13 and plasmid DNA. Bacteria were

plated on H agar and grown up overnight at 37°C. A single colony was picked and grown up in 10 mls Luria broth by shaking for at least 7 h at 37°C. This culture could be stored at 4°C for several days or for long term storage at -70°C in 15% glycerol. A fresh culture was prepared by scraping the surface of the frozen stock and inoculating into 10mls Luria broth and growing up as before. The concentration of cells in a culture was estimated by measuring the O.D.<sub>600nm</sub>, where 1 O.D. =  $8 \times 10^8$  cells.

#### V. Organosilanation of slides.

Slides were treated with organosilane to prevent loss of sections during *in situ* hybridisation treatments.

##### (a) Slide preparation.

Slides were washed in 1M HCl for 20 mins, rinsed briefly in dH<sub>2</sub>O, washed in absolute alcohol and wiped clean with a tissue. The slides were coated overnight in 1X Denhardt's solution (1X = 0.02% BSA, 0.02% PVP, 0.02% Ficoll). The slides were rinsed briefly in dH<sub>2</sub>O, fixed in ethanol:acetic acid (3:1) for 20 mins at room temperature, and air dried. They were then acetylated as follows: slides were immersed in 0.1M triethanolamine pH 8.0 and acetic anhydride was added to a final concentration of 0.25% (v/v) and vigorously mixed for 10 mins. The slides were then washed in dH<sub>2</sub>O for 10 mins.

##### (b) Organosilanation.

Slides were incubated overnight in a 1% (v/v) solution of organosilane (gamma-aminopropyl-triethoxy-silane) pH 3.45 at 70°C, washed extensively in dH<sub>2</sub>O, air dried and baked at 100°C overnight. Silanated slides were stored until required (for up to 6 months) in dust-free boxes at room temperature.

(c) Slide activation.

Slides were activated by incubation in a 10% (v/v) solution of glutaraldehyde in PBS'A' at pH 7.0 for 30 mins at room temperature. They were briefly rinsed with dH<sub>2</sub>O, then stabilised by treatment with 0.1M sodium metaperiodate for 15 mins, rinsed with PBS'A' and dehydrated in graded alcohols. Dried slides were stored at room temperature for up to 2 weeks.

**VI. Growth of paramyxovirus strains.**

Stocks of the paramyxoviruses MV, CDV and SV5 were prepared as follows. Defective-interfering (DI) virus particles may be produced during infection by paramyxoviruses. These interfere with replication of the normal virus particles, and hence reduce viral titres. The following procedure was therefore undertaken to reduce the levels of DI particles in the virus stocks.

(a) Preparation of master stocks.

Monolayers of African Green monkey kidney (Vero) cells were prepared in a 96-well microtitre plate by spotting  $2 \times 10^4$  cells per well from a trypsinised cell suspension in Dulbecco's modification of Eagle's tissue culture medium supplemented with 10% calf serum, and incubated overnight at 37°C in a CO<sub>2</sub>-gassed incubator. A number of 25cm<sup>2</sup> flats were prepared by seeding  $2 \times 10^6$  cells per flask and incubating at 37°C. When the monolayers in the 96 well plate were confluent, the medium was removed and replaced with virus diluted in medium. For all infections the amount of calf serum in the medium was reduced to 2%. Two-fold serial dilutions of virus were made across each row of the plate, with a final volume of 150µl medium per well. The cells were incubated at 37°C until an extensive cytopathic effect (c.p.e.) was seen. The wells with the lowest dilution of virus which showed 100% c.p.e. were used to infect the 25cm<sup>2</sup> flasks. The medium was removed from each flask and replaced with 150µl virus dilution in 1ml medium. Virus was

adsorbed for 1-2 h, then flooded and incubated until an extensive c.p.e. was seen. A number of 75cm<sup>2</sup> flasks were prepared by seeding with approximately  $5 \times 10^6$  cells per flask. The 25cm<sup>2</sup> flasks showing the most extensive c.p.e. were used to infect the 75cm<sup>2</sup> flasks. One millilitre of virus was added with 2 mls medium and adsorbed for 1-2 h, then flooded with 3-4 mls medium and incubated until an extensive c.p.e. was seen. This virus solution was used to infect two Burrlers, prepared by seeding with cells from two confluent 75cm<sup>2</sup> flasks in a volume of 250 mls medium and incubating until confluent. Two mls of virus was added to each Burrler in 25mls medium and adsorbed for 1-2 h. A further 25mls of medium was added and the cells incubated until small plaques were just visible. The medium was discarded and replaced with 30mls fresh medium and incubated until approximately 100% of the monolayer showed a c.p.e. The medium was harvested and stored in aliquots at -70°C. This master stock was used to grow up working stocks.

(b) Preparation of a working virus stock.

A confluent monolayer of Vero cells in a 75cm<sup>2</sup> flask was infected with 100μl of virus stock in 3mls medium supplemented with 2% calf serum and adsorbed at 37°C for 1-2 h. A further 2mls was added and the cells incubated until an extensive c.p.e. was seen. The medium was harvested and centrifuged at 1,500 rpm for 10 mins to remove cell debris. The virus stock was stored at -70°C in aliquots until required.

The virus dilution required to give rise to individual plaques on a monolayer was determined by infection of monolayers on a multiwell slide with different virus dilutions. A more accurate determination of virus titres was not usually necessary.

Table I. Media for growth of bacteria.

2X TYE	16g tryptone 10g yeast extract 5g NaCl
Luria broth	10g tryptone 5g yeast extract 5g NaCl
H agar	10g tryptone 8g NaCl 15g agar
H-top agar	10g tryptone 8g NaCl 7g agar

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| All solutions made up to 1 litre with distilled water and autoclaved. |  |



Table II. Solutions for transformation of *E.coli*.

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SOB	2.5mM KCl 10mM NaCl 2% (w/v) Bactotryptone 0.5% (w/v) yeast extract 10mM MgCl <sub>2</sub> 10mM MgSO <sub>4</sub>
SOC	SOB + 10mM sterile glucose
TFB	10mM K-Mes (morpholino-ethane sulphonic acid) pH 6.3 100mM KCl 45mM MnCl <sub>2</sub> 10mM CaCl <sub>2</sub> 3mM hexamine cobalt chloride

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| All solutions were sterilised and stored at 4°C. |  |

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Table III. Solutions for forward-labelling of M13 probes.

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M13 TE buffer.	10mM Tris-HCl pH 8.0 0.1mM EDTA
TM buffer	100mM Tris-HCl pH 8.0 100mM MgCl <sub>2</sub>
CGT mix	Add equal volumes of : 0.5mM dATP 0.5mM dGTP 0.5mM dTTP TE buffer
10X EcoRI digestion buffer	1M NaCl 0.5M Tris-HCl pH 7.5 0.1M MgCl <sub>2</sub> 10mM DTT
10X HindIII digestion buffer	0.5M NaCl 0.1M Tris-HCl pH 7.5 0.1M MgCl <sub>2</sub> 10mM DTT
5X loading buffer	12.5% Ficoll 50mM EDTA 0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol

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Table IV. Solutions for purification of plasmid DNA.

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STE buffer	100mM NaCl 20mM Tris-HCl pH 7.8 5mM EDTA
Solution I	50mM glucose 25mM Tris-HCl pH 7.8 10mM EDTA 5mg/ml lysozyme (added fresh)
Solution II	0.2M NaOH 1% SDS
Solution III	5M potassium acetate
TE buffer	100mM Tris-HCl pH 8.0 1mM EDTA
TEN buffer	100mM NaCl 10mM Tris-HCl pH 8.0 1mM EDTA

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Table V. Solutions for *in vitro* transcription of RNA probes.

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DEPC-treated water	DEPC added to 0.2 % and autoclaved.
10X transcription buffer	200mM Tris-HCl pH 7.2 30mM MgCl <sub>2</sub>
5X XTP mix	2mM CTP 2mM GTP 2mM ATP
5X -S-UTP	80umα-S-UTP

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| All solutions were prepared in DEPC-treated water. |  |

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**REFERENCES.**



Ablashi,D.V., Easton,J.M. & Guegan,J.H. 1976. Herpesviruses and cancer in man and subhuman primates. *Biomedicine* **24**: 286-305.

Abraham,G. & Banerjee,A.K. 1976. Sequential transcription of the genes of vesicular stomatitis virus. *Proceedings of the National Academy of Sciences (USA)* **73**: 1504-1508.

Al-Hakim,A.H. & Hull,R. 1988. Chemically synthesised non-radioactive biotinylated long-chain nucleic acid hybridisation probes. *Biochemical Journal* **251**: 935-938.

Anderson,R.E. & Standefer,J.C. 1983. Radiation injury in the immune system. In "Cytotoxic insult to tissue. Effects on cell lineages". Eds. Potten,C.S. & Hendry,J.H. Churchill Livingstone. pp67-104.

Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.F., Jonat,P., Herrlich,C. & Karin,M. 1987. Phorbol ester-inducible genes contain a common cis element recognised by a TPA-modulated trans-acting factor. *Cell* **49**: 729-739.

Angerer,L.M. & Angerer,R.C. 1981. Detection of poly-A RNA in sea urchin eggs and embryos by quantitative *in situ* hybridisation. *Nucleic Acids Research* **9**: 2819-2840.

Andjaparidze,O.G., Chaplygina,N.M., Bogomolova,N.N., Koptyaeva,I.B., Nevryaeva,E.G., Filimonova,R.G. & Tareeva,I.E. 1989. Detection of measles virus genome in blood leucocytes of patients with certain autoimmune diseases. *Archives of Virology* **105**: 287-291.

Assaad,F. 1983. Measles : summary of worldwide impact. *Reviews of Infectious Diseases* **5**: 452-459.

Axthelm,M.K. & Krakowka,S. 1986. Immunocytochemical methods for demonstrating canine distemper virus antigen in aldehyde-fixed paraffin-embedded tissue. *Journal of Virological Methods* **13**: 215-229.

Baczko,K., Liebert,U.G., Billeter,M.A., Cattaneo,R., Budka,H. & ter Meulen,V. 1986. Expression of defective measles virus genes in brain tissue of patients with subacute sclerosing panencephalitis. *Journal of Virology* **59**: 472-478.

Bagasra,O. 1990. In "Amplifications : A forum for PCR users". Perkin Elmer Cetus.

Baker,J.R. 1960. Principles of Biological Microtechnique. Methuen : London.

Baldino,F. & Lewis,M.E. 1989. Nonradioactive *in situ* hybridisation histochemistry with digoxigenin-deoxyuridine 5'-triphosphate-labelled oligonucleotides. In "Methods in Neurosciences". Volume 1. pp282-292.

Ball,L.A. & White,C.N. 1976. Order of transcription of genes of vesicular stomatitis virus. Proceedings of the National Academy of Sciences (USA) **73**: 442-446.

Bangham,C.R.M., Cannon,M.J., Karzon,D.T. & Askonas,B.A. 1985. Cytotoxic T-cell response to respiratory syncytial virus in mice. Journal of Virology **56**: 55-59.

Bankier,A.T., Dietrich,W., Baer,R., Barrell,B.G., Colbere-Garapin,F., Fleckenstein,B. & Bodemer,W. 1985. Terminal repetitive sequences in herpesvirus saimiri virion DNA. Journal of Virology **55**: 133-139.

Barker,D.J., Clough,P.W., Guyer,P.B. & Gardner,M.J. 1977. Paget's disease of bone in 14 British towns. British Medical Journal **1**: 1181-1183.

Barker,D.J., Chamberlain,A.T., Guyer,P.B. & Gardner,M.J. 1980. Paget's disease of bone : the Lancashire focus. British Medical Journal **280**: 1105-1107.

Basle,M.F., Fournier,J.G., Rozenblatt,S., Rebel,A. & Bouteille,M. 1986. Measles virus RNA detected in Paget's disease of bone tissue by *in situ* hybridisation. Journal of General Virology **67**: 907-913.

Basle,M.F., Rebel,A., Fournier,J.G., Russell,W.C. & Malkani,K. 1987. On the trail of paramyxoviruses in Paget's disease of bone. Clinical Orthopaedics and Related Research **217**: 9-15.

Basle,M., Rebel,A., Pouplard,A., Kongourdnjian,S., Filman,R. & Lepalezour,A. 1979. Demonstration of measles virus antigens in osteoclasts in Paget's disease of bone. C.R.Academy of Science (Paris) **289**: 225-228.

Basle,M.F., Russell,W.C., Goswami,K.K.A., Rebel,A., Virandon,P., Wild,F. & Filman,R. 1985. Paramyxovirus antigens in osteoclasts from Paget's bone tissue detected by monoclonal antibodies. Journal of General Virology **66**: 2103-2110.

- Bass,B.L. & Weintraub,H. 1988. An unwinding activity that covalently modifies its double-stranded RNA substrate. *Cell* **55**: 1089-1098.
- Bauman,J.G.J. 1985. Fluorescence microscopical hybridocytochemistry. *Acta Histochemica* **31**: 9-18.
- Berger,C.N. 1986. *In situ* hybridisation of immunoglobulin-specific RNA in single cells of the B lymphocyte lineage with radiolabelled DNA probes. *EMBO Journal* **5**: 85-93.
- Bhatt,B., Burns,J., Flannery,D. & McGee,J. 1988. Direct visualisation of single copy genes on banded metaphase chromosomes by nonisotopic *in situ* hybridisation. *Nucleic Acids Research* **16**: 3951-3961.
- Biggin,K., Farrell,P.J. & Barrell,B.G., 1984. Transcription and DNA sequence of the BamHI L fragment of B95-8 Epstein-Barr virus. *EMBO Journal* **3**: 1083-1090.
- Binder,M., Tourmente,S., Roth,J., Renaud,M. & Gehring,W. 1986. *In situ* hybridisation at the electron microscope level : Localisation of transcripts on ultrathin sections of lowicryl K4M-embedded tissue using biotinated probes and protein A-gold complexes. *Journal of Cell Biology* **120**: 1646-1653.
- Bittle,J.L., Houghton,R.A., Alexander,H., Shinnick,T.M., Sutcliffe,J.G., Lerner,R.A., Rowlands,D.J. & Brown,F. 1982. Protection against foot-and-mouth disease by immunisation with a chemically synthesised peptide predicted from the viral nucleotide sequence. *Nature (London)* **298**: 30-33.
- Black,F.L. 1988. Persistent measles virus genome in autoimmune chronic active hepatitis : cause or coincidence? *Hepatology* **8**: 186-187.
- Blair,E.D. & Honess,R.W. 1983. DNA-binding proteins specified by herpesvirus saimiri. *Journal of General Virology* **64**: 2697-2715.
- Blum,H.E., Haase,A.T. & Vyas,G.N. 1984. Molecular pathogenesis of hepatitis B virus infection : Simultaneous detection of viral DNA and antigens in paraffin-embedded liver sections. *Lancet* **i**: 771-774.

Bonner,T.I., Brenner,D.I., Neufeld,B.R. & Britten,R.J. 1973. Reduction in the rate of DNA reassociation by sequence divergence. *Journal of Molecular Biology* **81**: 123-135.

Bos,J.L, Velaan-de Vries,M., Jansen,A.M., Veeneman,G.H., van Boom,J.H. & van der Eb,A.J. 1984. Three different mutations in codon 61 of the human N-ras gene detected by synthetic oligonucleotide hybridisation. *Nucleic Acids Research* **12**: 9155-9163.

Brahic,M. & Haase,A.T. 1978. Detection of viral sequences of low reiteration frequency by *in situ* hybridisation. *Proceedings of the National Academy of Sciences (USA)* **75**: 6125-6129.

Brahic,M., Haase,A.T. & Cash,E. 1984. Simultaneous *in situ* detection of viral RNA and antigens. *Proceedings of the National Academy of Sciences (USA)* **81**: 5445-5448.

Brahic,M., Stowring,L., Ventura,P. & Haase A.T. 1981. Gene expression in visna virus infection. *Nature (London)* **292**: 240-242.

Brahic,M. & Stroop,W.G. 1981. Theiler's virus persists in glial cells during demyelinating disease. *Cell* **26**: 123-128.

Brigati,D.J., Myerson,D. Leary,J.J., Spalholtz,B. Travis,S.Z., Fong,C.K.Y., Hsiung,G.D. & Ward,D.C. 1983. Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labelled hybridisation probes. *Virology* **126**: 32-50.

Burns,J., Chan,V.T-W., Jonasson,J.A., Fleming,K.A., Taylor,S. & McGee,J.O'D. 1985. A sensitive method for visualising biotinylated probes hybridised *in situ* : rapid sex determination on intact cells. *Journal of Clinical Pathology* **38**: 1085-1092.

Burns,J., Redfern,D.R.M., Esiri,M.M., McGee,J.O'D. 1986. Human and viral gene detection in routine paraffin-embedded tissue by *in situ* hybridisation with biotinylated probes : viral localisation in herpes encephalitis. *Journal of Clinical Pathology* **40**: 163-166.

Butler,E. & Chamberlain,M.J. 1982. Bacteriophage SP6-specific RNA polymerase I. Isolation and characterisation of the enzyme. *Journal of Biological Chemistry* **257**: 5772-5778.

Buvoli,M., Biamonti,G. & Morandi,C. 1987. Hybridisation of oligodeoxynucleotide probes to RNA molecules : specificity and stability of duplexes. *Nucleic Acids Research* **15**: 9091.

Cameron,K.R., Stamminger,T., Craxton,M., Bodemer,W., Honess,R.W. & Fleckenstein,B. 1987. The 160,000-Mr virion protein encoded at the right end of the herpesvirus saimiri genome is homologous to the 140,000-Mr membrane antigen encoded at the left end of the EBV genome. *Journal of Virology* **61**: 2063-2070.

Cannon,M.J., Stott,E.J., Taylor,G. & Askonas,B.A. 1987. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. *Immunology* **62**: 133-138.

Cantrill, J.A., Buckler,H.M. & Anderson,D.C. 1986. Low dose intravenous 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (APD) for the treatment of Paget's disease of bone. *Annual Rheumatic disease* **45**: 1012-1218.

Cantrill, J.A., Buckler,H.M. & Anderson,D.C. 1988. Primary treatment of 133 patients with Paget's disease of bone with intravenous 3-amino-hydroxypropylidene-1,1-bisphosphonate (APD). *Calcified Tissue International*, Supplement **42**: Abstract 52.

Carrigan,D.R. & Kabacoff,C.M. 1987. Identification of a non-productive cell-associated form of measles virus by its resistance to inhibition by recombinant human interferon. *Journal of Virology* **61**: 1919-1926.

Casali,P., Rice,G.P.A. & Oldstone,M.B.A. 1984. Viruses disrupt the functions of human lymphocytes. Effects of measles virus and influenza virus on lymphocyte-mediated killing and antibody production. *Journal of Experimental Medicine* **159**: 1322-1337.

Casey,J. & Davidson,N. 1977. Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide. *Nucleic Acids Research* **4**: 1539-1552.



Cattaneo,R., Schmid,A., Eachle,D., Baczko,K., ter Meulen,V. & Billeter,M.A. 1988. Biased hypermutation and other genetic changes in defective measles viruses in human brain infections. *Cell* **55**: 255-265.

Cattaneo,R., Schmidt,A., Rebmann,G., Baczko,K., ter Meulen,V., Bellini,W.J., Rozenblatt,S. & Billeter,M.A. 1986. Accumulated mutations in a case of subacute sclerosing panencephalitis : interrupted matrix protein reading frame and transcription alteration. *Virology* **154**: 97-107.

Chang,P.W. & Hsiung,G.D. 1965. Experimental infection of parainfluenza virus type 5 in mice, hamsters and monkeys. *Journal of Immunology* **95**: 591-601.

Chevallier-Greco,A., Manet,E., Chavrier,P., Mosnier,C., Dallie,J. & Sergeant,A. 1986. Both Epstein-Barr virus (EBV)-encoded transacting factors, EB1 and EB2 are required to activate transcription from an EBV early promoter. *EMBO Journal* **5**: 3243-3249.

Choppin,P.W. & Scheid,A. 1980. The role of viral glycoproteins in adsorption, penetration and pathogenicity of viruses. *Reviews of Infectious Diseases* **2**: 40-61.

Christie,K.E. & Hawkenes,G. 1983. Measles virus-specific IgM antibodies in sera from patients with chronic active hepatitis. *Journal of Medical Virology* **12**: 267-272.

Church,G.M. & Gilbert,W. 1984. Genomic sequencing. *Proceedings of the National Academy of Sciences (USA)* **81**: 1991-1995.

Coglan,J.P., Penschow,J.D., Fraser,J.R., Aldred,P., Haralambidis,J. & Tregear,G.W. 1987. Location of gene expression in mammalian cells. In " *In situ* hybridisation : Applications to Neurobiology". Eds. Valentino,K.L., Eberwine,J.H. & Barchas,J.D. Oxford University Press : New York. pp25-41.

Cohen,G.H., Dietzschold,B., Ponce de Leon,M., Long,D., Golub,E., Varrichio,A., Pereira,L. & Eisenberg,R.J. 1984. Localisation and synthesis of an antigenic determinant of herpes simplex virus glycoprotein D that stimulates the production of neutralising antibodies. *Journal of Virology* **49**: 102-108.

Cohen,M.L., Golde,T.E. Usiak,M.F., Younkin,L.H. & Younkin,S.G. 1988. *In situ* hybridisation of nucleus basalis neurons shows increased  $\beta$ -amyloid mRNA in Alzheimer disease. *Proceedings of the National Academy of Sciences (USA)* **85**: 1227-1231.

Collins,D.H. 1956. Paget's disease of bone : incidence and sub-clinical forms. *Lancet* **ii**: 51-57.

Collins,M.L. & Hunsaker,W.R. 1985. Improved hybridisation assays employing tailed oligonucleotide probes : a direct comparison with 5'-end-labelled oligonucleotide probes and nick-translated plasmid probes. *Analytical Biochemistry* **151**: 211-224.

Colonno,R.J. & Banerjee,A.K. 1977. Mapping and initiation studies on the leader RNA of vesicular stomatitis virus. *Virology* **77**: 260-268.

Connolly,J.H. 1968. Additional data on measles virus antibody and antigen in subacute sclerosing panencephalitis. *Neurology* **18**: 87-89.

Connolly,J.H., Allen,I.V., Hurwitz,L.J. & Miller,J.H.D. 1967. Measles virus antibody and antigen in subacute sclerosing panencephalitis. *Lancet* **i**: 542.

Cosby,S.L., McQuaid,S., Taylor,M.J., Bailey,M., Rima,B.K., Martin S.J. & Allen,I.B. 1989. Examination of eight cases of multiple sclerosis and fifty-six neurological and non-neurological controls for genomic sequences to measles virus, canine distemper virus, simian virus 5 and rubella virus. *Journal of General Virology* **70**: 2027-2036.

Costanzo,F., Campadelli-Fiume,G., Foa-Tomasi,L. & Cassali,E. 1977. Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. *Journal of Virology* **21**: 996-1001.

Countryman,J., Jenson,H., Seibl,R., Wolf,H. & Miller,G. 1987. Polymorphic proteins encoded within BZLF1 of defective and standard Epstein-Barr viruses disrupt latency. *Journal of Virology* **61**: 3672-3679.

Countryman,J. & Miller,G. 1985. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. *Proceedings of the National Academy of Sciences (USA)* **82**: 4085-4089.

- Cowdry,E.B. 1934. The problem of intranuclear inclusions in virus diseases. Archives of Pathology 18: 527-542.
- Cox,K.H., Deleon,V., Angerer,L.M. & Angerer,R.C. 1984. Detection of mRNAs in sea urchin embryos by *in situ* hybridisation using asymmetric RNA probes. Developmental Biology 101: 485-502.
- Croen,K.D., Ostrove,J.M., Dragovic,L.J., Smialek,J.E. & Strauss,S.E. 1987. Latent herpes simplex virus in human trigeminal ganglia : detection of an immediate-early gene "anti-sense" transcript by *in situ* hybridisation. New England Journal of Medicine 317: 1427-1432.
- Cubie,H.A. & Norval,M. 1989. Detection of human papillomaviruses in paraffin wax sections with biotinylated synthetic oligonucleotide probes and immunogold staining. Journal of Clinical Pathology 42: 988-991.
- Cunningham,M.W. & Mundy,C.R. 1987. Labelling nucleic acids for hybridisation. Nature (London) 326: 723-724.
- Daniel,M.D., Melendez,L.V., Hunt,R.D., King,N.W., Anver,M., Fraser,C.E.O., Barahona,H.H. & Baggs,R.B. 1974. Herpesvirus saimiri VII. Induction of malignant lymphoma in New Zealand white rabbits. Journal of the National Cancer Institute 53: 1803-1807.
- Davison,A.J. & Scott,J.E. 1986. The complete DNA sequence of varicella-zoster virus. Journal of General Virology 67: 1759-1816.
- Dawson,J.R.Jr. 1933. Cellular inclusions in cerebral lesions of lethargic encephalitis. American Journal of Pathology 9: 7-15.
- Deinhardt,F., Falk,L. & Wolfe,L.G. 1973. Simian herpesviruses. Cancer Research 33: 1424-1426.
- Desrosiers,R.C., Silva,D.P., Waldron,L.M. & Letvin,N.L. 1986. Nononcogenic deletion mutants of herpesvirus saimiri are defective for *in vitro* immortalisation. Journal of Virology 57: 701-705.

DeVries,P., Binnendijk,R.S.V., van der Marel,P., van Wezel,A.L., Voorma,H.O., Sundquist,B., Uytdehaag,F.G.C.M. & Osterhaus,A.D.M.E. 1988. Measles virus fusion protein presented in an immune-stimulating complex (Iscom) induces haemolysis-inhibiting and fusion-inhibiting antibodies, virus-specific T cells and protection in mice. *Journal of General Virology* **69**: 549-559.

Diaz,M., Barsacchi-Pilone,G., Mahon,K. & Gall,J. 1981. Transcripts from both strands of a satellite DNA occur on lampbrush chromosome loops of the newt *Notophthalmus*. *Cell* **24**: 649-659.

Dowling,P.C., Blumberg,B.M., Kolakofsky,D., Cook,P., Jotkowitz,A. Prineas,J.W. & Cook,S.D. 1986. Measles virus nucleic acid sequences in human brain. *Virus Research* **5**: 97-107.

Drillien,R., Spehner,D., Kirn,A., Giraudon,P., Buckland,R., Wild,F. & Lecocq,J-P. 1988. Protection of mice from fatal measles encephalitis by vaccination with vaccinia virus recombinants encoding either the haemagglutinin or the fusion protein. *Proceedings of the National Academy of Sciences USA* **85**: 1252-1256.

Emerson,S.U. 1982. Reconstitution studies detect a single polymerase entry site on the vesicular stomatitis virus genome. *Cell* **31**: 635-642.

Emerson,S.U. 1988. Rhabdoviruses. In "Virology". Ed. Fields,B.N. & Knipe,D.M. Raven Press: New York. pp477-490.

Evans,D.J., McKeating,J., Meredith,J.M., Burke,K.L., Katrak,K., John,A., Ferguson,M., Minor,P.D., Weiss,R.A. & Almond,J.W. 1989. An engineered poliovirus chimaera elicits broadly reactive HIV-1 neutralising antibodies. *Nature (London)* **339**: 385-388.

Falk,L.A., Nigida,S., Deinhardt,F., Cooper,R.W. & Hernandez-Camacho,J.I. 1973. Oral excretion of herpesvirus saimiri in captive squirrel monkeys and incidence of infection in feral squirrel monkeys. *Journal of the National Cancer Institute* **51**: 1987-1989.

Falk,L.A., Wolfe,L.G., Hoekstra,J. & Deinhardt,F. 1972. Isolation of herpesvirus saimiri from blood of squirrel monkeys (*Saimiri sciureus*). *Journal of the National Cancer Institute* **48**: 1499-1505.

Fallon,M.C. 1983. Osteoblasts mediate osteoclastic bone resorption in cell culture. *Calcified Tissue International* **35**: 640.

Farrell,P., Rowe,D., Rooney,C. & Konzarides,J.T. 1989. Epstein-Barr virus BZLF1 trans-activator specifically binds to consensus AP-1 site and is related to C-Fos. *EMBO Journal* **8**: 127-132.

Feinberg,A.P. & Vogelstein,B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**: 6-13.

Fitzpatrick-McElligott,S., Lewis,M.E., Tyler,M., Baldino,F. & Davis,L.G. 1988. *In situ* hybridisation with radiolabelled, synthetic oligodeoxyribonucleotide probes. In "Biotech Update". Du Pont.

Forster,A.C., McInnes,J.L., Skingle,D.C. & Symons,R.H. 1985. Non-radioactive hybridisation probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. *Nucleic Acids Research* **13**: 745-761.

Fournier,J-G., Rozenblatt,S. & Bouteille,M. 1983. Localisation of measles virus nucleic acid sequences in infected cells by *in situ* hybridisation. *Biology of the Cell* **49**: 287-290.

Fournier,J-G., Tardieu,M., Lebon,P., Robain,O.,Ponsot,G., Rozenblatt,S. & Bouteille,M. 1985. Detection of measles virus RNA in lymphocytes from peripheral blood and brain perivascular infiltrates of patients with sub-acute sclerosing panencephalitis. *New England Journal of Medicine* **313**: 910-915.

Friedman,R.M. & Ramseur,J.M. 1979. Mechanisms of persistent infections by cytopathic viruses in tissue culture. *Archives of Virology* **60**: 83-103.

Fujinami,R.S. Sissons,J.G.P. & Oldstone,M.B.A. 1981. Immune reactive measles virus polypeptides on the cell's surface : Turnover and relationship of glycoproteins to each other and to HLA determinants. *Journal of Immunology* **127**: 935-940.

Fujinami,R.S. & Oldstone,M.B.A. 1979. Antiviral antibody on the plasma membrane alters measles virus expression inside the cell. *Nature (London)* **279**: 529-530.



- Fujinami, R.S. & Oldstone, M.B.A. 1980. Alterations in expression of measles virus polypeptides by antibody : Molecular events in antibody-induced antigenic modulation. *Journal of Immunology* **125**: 78-85
- Fulginiti, V.A., Eller, J.J., Sieber, O.F., Joyner, J.W., Minamitani, M. & Melklejohn, O. 1969. Respiratory virus immunisation. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. *American Journal of Epidemiology* **89**: 435-448.
- Gall, J.G. & Pardue, M.L. 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proceedings of the National Academy of Sciences (USA)* **63**: 378-383.
- Gall, J.G. & Pardue, M.L. 1971. Nucleic acid hybridisation in cytological preparations. In "Methods in Enzymology" Volume XXI. Eds. Grossman, L. & Moldave, K. Academic Press : New York. pp470-480.
- Gallagher, W.R. & Bratt, M.A. 1974. Conditional dependence of fusion from within and other cell membrane alteration by Newcastle disease virus. *Journal of Virology* **14**: 813-820.
- Galloway, D.A., Fenoglio, C.M. & McDougall, J.K. 1982. Limited transcription of the herpes simplex virus genome when latent in human sensory ganglia. *Journal of Virology* **41**: 686-691.
- Gavish, D., Kleinman, Y., Morag, A. & Chejek-Shaul, T. 1983. Hepatitis and jaundice associated with measles in young adults. *Archives of Internal Medicine* **143**: 674-677.
- Gee, C.E. & Roberts, J.L. 1983. *In situ* hybridisation histochemistry : A technique for the study of gene expression in single cells. *DNA* **2**: 157-163.
- Gerhard, D.S., Kawasaki, E.S., Carter, B.F. & Szabo, P. 1981. Localisation of a unique gene by direct hybridisation *in situ*. *Proceedings of the National Academy of Sciences (USA)* **78**: 3755-3759.
- Gheradi, G., Caschio, V. & Bonnucci, E. 1980. Fine structure of nuclei and cytoplasm of osteoclasts in Paget's disease of bone. *Proceedings of the National Academy of Sciences (USA)* **77**: 3686-3690.

Giraudon,P. & Wild,T.F. 1985. Correlation between epitopes on haemagglutinin of measles virus and biological activities : passive protection by monoclonal antibodies is related to their haemagglutinin activity. *Virology* **144**: 46-58.

Godard,C.M. 1983. Improved method for detection of cellular transcripts by *in situ* hybridisation : Detection of virus-specific RNA in Rous sarcoma virus-infected cells by *in situ* hybridisation to cDNA. *Histochemistry* **77**: 123-131.

Gompels,U.A., Craxton,M.A. & Honess,R.W. 1988. Conservation of gene organisation in the lymphotropic herpesviruses herpesvirus saimiri and Epstein-Barr virus. *Journal of Virology* **62**: 757-767.

Gorden,Y.J., Johnson,B., Romanowski,E. & Aravello-Cruz,T. 1988. RNA complementary to herpes simplex virus type 1 ICP0 gene demonstrated in neurons of human trigeminal ganglia. *Journal of Virology* **62**: 1832-1835.

Goswami,K.K.A., Cameron,K.R., Russell,W.C., Lange,L.S. & Mitchell,D.N. 1984. Evidence for persistence of paramyxoviruses in human bone marrows. *Journal of General Virology* **65**: 1881-1888.

Grogan,E., Jenson,H., Countryman,J., Heston,L., Gradoville,L. & Miller,G. 1987. Transfection of a rearranged viral DNA fragment, WZhet, stably converts Epstein-Barr viral infection to productive infection in lymphoid cells. *Proceedings of the National Academy of Sciences (USA)* **84**: 1332-1336.

Guitteny,A-F., Bohlen,P. & Bloch,B. 1988. Analysis of vasopressin gene expression by *in situ* hybridisation and immunohistochemistry in semi-thin sections. *Journal of Histochemistry & Cytochemistry* **36**: 1374-1378.

Haase,A.T. 1986. Analysis of viral infections by *in situ* hybridisation. *Journal of Histochemistry and Cytochemistry* **34**: 27-32.

Haase,A.T. 1987. Analysis of viral infections by *in situ* hybridisation. In "*In situ* hybridisation : Applications to neurobiology." Eds. Valentino,K., Roberts,J. & Barchas,J. Oxford University Press : New York. pp197-219.

Haase,A.T., Brahic,M., Stowring,L. & Blum,H. 1984a. Detection of viral nucleic acids by *in situ* hybridisation. *Methods in Virology VII*: 189-226.

Haase,A.T., Gantz,D., Blum,H., Stowring,L., Ventura,P., Geballe,A., Moyer,B. & Brahic,M. 1985a. Combined macroscopic and microscopic detection of viral genes in tissues. *Virology* **140**: 201-206.

Haase,A.T., Gantz,D., Eble,B., Walker,D., Stowring,L., Ventura,P., Blum,H., Wietgreffe,S., Zupancic,M., Tourtelotte,W., Gibbs,C.J., Norrby,E. & Rozenblatt,S. 1985b. Natural history of restricted synthesis and expression of measles virus genes in sub-acute sclerosing panencephalitis. *Proceedings of the National Academy of Sciences (USA)* **82**: 3020-3024.

Haase,A.T., Pagano,J., Waksman,B. & Nathanson,N. 1984b. Detection of viral genes and their products in chronic neurological diseases. *Annals of Neurology* **15**: 119-121.

Haase,A.T. & Stowring,L. 1977. Slow persistent infection caused by Visna virus : Role of host restriction. *Science* **195**: 175-177.

Haase,A.T., Swoveland,P., Stowring,L., Ventura,P., Johnson,K.P., Norrby,E. & Gibbs,Jr.C.J. 1981a. Measles virus genome in infections of the central nervous system. *Journal of Infectious Diseases* **144**: 154-160.

Haase,A.T., Ventura,P., Gibbs,C.J. & Tourtellotte,W.W. 1981b. Measles virus nucleotide sequences : detection by hybridisation *in situ*. *Science* **212**: 672-674.

Haase,A.T., Walker,D., Stowring,L., Ventura,P., Geballe,A., Blum,H., Brahic,M., Glodberg,R. & O'Brien,K. 1985c. Detection of two viral genomes in single cells by double-label hybridisation *in situ* and colour microradioautography. *Science* **227**: 189-192.

Hall,B. 1972. Differentiation of cartilage and bone from common germinal cells. *Calcified Tissue Research* **8**: 276-286.

Hall,W.W. & Choppin,P.W. 1979. Evidence for the lack of synthesis of the M polypeptide of measles virus in brain cells from SSPE. *Virology* **99**: 443-447.

Hall,W.W., Lamb,R.A. & Choppin,P.W. 1979. Measles and sub-acute sclerosing panencephalitis virus proteins : lack of antibodies to the M protein in patients with sub-acute sclerosing panencephalitis. *Proceedings of the National Academy of Sciences (USA)* **76**: 2047-2051.

Hamdy,R.C. 1981. Paget's disease of bone. New York : Praeger.

Hamill,R.J., Baughn,R.E., Mallette,L.E., Musher,D.M. & Wilson,D.B. 1986. Serological evidence against a role for canine distemper virus in pathogenesis of Paget's disease of bone. *Lancet* **1**: 1399.

Hanahan,D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**: 557-580.

Harper,M.E., Ulrich,A. & Saunders,G.R. 1981. Localisation of the human insulin gene to the distal end of the short arm of chromosome 11. *Proceedings of the National Academy of Sciences (USA)* **78**: 4458-4460.

Harvey,L., Gray,T., Beneton,M.N.C., Douglas,D.L., Kanis,J.A. & Russell,R.G.G. 1982. Ultrastructural features of the osteoclasts from Paget's disease of bone in relation to a viral aetiology. *Journal of Clinical Pathology* **35**: 771-779.

Haspel,M.V., Duff,R.G. & Rapp,F. 1975. The isolation and preliminary characterisation of temperature-sensitive mutants of measles virus. *Journal of Virology* **16**: 1000-1009.

Hayashi,S., Gillam,I.C., Delaney,A.D. & Tener,G.M. 1978. Acetylation of chromosome squashes of *Drosophila melanogaster* decreases the background in autoradiographs with [<sup>125</sup>I]-labelled RNA. *Journal of Histochemistry and Cytochemistry* **26**: 677-679.

Hiebert,S.W., Paterson,R.G. & Lamb,R.A. 1985. Haemagglutinin-neuraminidase protein of the paramyxovirus simian virus 5 : Nucleotide sequence of the mRNA predicts an N-terminal membrane anchor. *Journal of Virology* **54**: 1-6.

Holland,J.J., Villareal,L.P., Welsh,R.M., Oldstone,M.B.A., Kohne,D., Lazzarini,R. & Scolnick,E. 1976. Long term persistent vesicular stomatitis virus and rabies virus infection of cells *in vitro*. *Journal of General Virology* **33**: 193.

Holland,J.J., Spindler,K., Horodyski,F., Graban,E., Nichol,S. & Vandepol,S. 1982. Rapid evolution of RNA genomes. *Science* **215**: 1577-1585.

Honess,R.W. & Roizman,B. 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *Journal of Virology* **14**: 8-19.

- Honess,R.W. & Roizman,B. 1975. Regulation of herpesvirus macromolecular synthesis : sequential transition of polypeptide synthesis requires functional viral polypeptides. *Proceedings of the National Academy of Sciences USA* **72**: 1276-1280.
- Hopman,A.H.N., Wiegant,J., Rapp,A.K., Landegent,J.E., van der Ploeg,M. & van Duijn,P. 1986. Bicolour detection of two target DNAs by non-radioactive *in situ* hybridisation. *Histochemistry* **85**: 1-4.
- Howatson,A.F. & Fornasier,V.L. 1982. Microfilaments associated with Paget's disease of bone. Comparison with nucleocapsids of measles virus and respiratory syncytial virus. *Intervirology* **18**: 150-159.
- Hutchinson,N.J., Langer-Safer,P.R., Ward,D.C. & Hamkalo,B.A. 1982. *In situ* hybridisation at the electron microscope level : hybrid detection by autoradiography and colloidal gold. *Journal of Cell Biology* **95**: 609-618.
- Ilonen,J., Ziola,B., Reunanen,M., Cantell,K., Paneluis,M., Lund,G. & Salmi,A. 1987. Immunological findings during an acute relapse of subacute sclerosing panencephalitis in a patient with an unusually prolonged disease course. *Journal of Clinical and Laboratory Immunology* **22**: 41-47.
- Ironside,J.W., Lewis,F.A., Blythe,D. & Wakefield,E.A. 1989. The identification of cells containing JC papovavirus DNA in progressive multifocal leukoencephalopathy by combined *in situ* hybridisation and immunocytochemistry. *Journal of Pathology* **157**: 291-297.
- Ito,Y., Nishiyama,Y., Shimokata,K., Kimura,Y., Nagata,I., Shimizu,K. & Kunii,A. 1975. Interferon induction in mice by BHK cells persistently infected with HVJ. *Journal of General Virology* **27**: 93-95.
- Jackson,D.P., Quirke,P., Lewis,F., Boylston,A.W., Sloan,J.M., Robertson,D. & Taylor,G.R. 1989. Detection of measles virus RNA in paraffin-embedded tissue. *Lancet* **i**: 1391.
- Jeffreys,A.J., Wilson,V. & Thein,S.L. 1985. Hypervariable 'minisatellite' regions in human DNA. *Nature (London)* **314**: 67-73.



Johnson,K.P., Norrby,E., Swoveland,P. & Carrogan,D.R. 1982. Expression of five viral antigens in cells infected with wild-type and SSPE strains of measles virus : correlation with cytopathic effects and productivty of infections. Archives of Virology 73: 255-262.

Joseph,B.S. & Oldstone,M.B.A.1974. Antibody-induced redistribution of measles virus antigens on the cell surface. Journal of Immunology 113: 1205-1209.

Joseph,B.S. & Oldstone,M.B.A. 1975. Immunologic injury in measles virus infection. II. Suppression of Immune Injury through antigenic modulation. Journal of Experimental Medicine 142: 864-876.

Kalland,K.H., Eridresen,C., Kaukenes,G. & Schrumpt,E. 1989. Measles-specific nucleotide sequences and autoimmune chronic active hepatitis. Lancet I: 1390-1391.

Kassavetis,G.A., Butler,E.T., Roulland,D. & Chamberlain,M.J. 1982. Bacteriophage SP6-specific RNA polymerase. II. Mapping of SP6 DNA and selective *in vitro* transcription. Journal of Biological Chemistry 257: 5779-5788.

Kim,H.W., Canchola,J.G., Brandt,C.D., Pyles,G. Chanock,R.M., Jenson,K & Parrott,R.H. 1969. Repsiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. American Journal of Epidemiology 89: 422-434.

Kimura,Y., Ito,Y., Shimokata,K., Nishiyama,Y., Nagata,I. & Kitoh,J. 1975. Temperature sensitive virus derived from BHK cells persistently infected with HVJ. Journal of Virology 15: 55.

Kingsbury,D.W., Bratt,M.A., Choppin,P.W., Hanson,R.P., Hosaka,Y., ter Meulen,V., Norrby,E., Plowright,W., Rott,R. & Wunner,W.H. 1978. Paramyxoviridae. Intervirology 10: 137-152.

Knight,P., Duff,R. & Rapp,F. 1972. Latency of human measles virus in hamster cells. Journal of Virology 10: 995-1001.

Knotts,F.B., Cook,M.L. & Stevens,J.G. 1973. Latent herpes simplex virus in the central nervous system of mice and rabbits. Journal of Experimental Medicine 138: 740-744.

Krane,S.M. 1977. Paget's disease of bone. *Clinical Orthopaedics & Related Research* **127**: 24-36.

Kumagi,T., Wong,D.T. & Ogra,P.L. 1985. Development of cell-mediated cytotoxic activity in the respiratory tract after experimental infection with respiratory syncytial virus. *Clinical and Experimental Immunology* **61**: 351-359.

Lagaris,D.M. & Radke,K. 1989. Transcriptional activation of bovine leukemia virus on blood cells from experimentally infected asymptomatic sheep with latent infections. *Journal of Virology* **63**: 2099-2107.

Lamb,R.A. & Dreyfuss,G. 1989. Unwinding with a vengeance. *Nature (London)* **337**: 19-20.

Langer,P.R., Waldrop,A.A. & Ward,D.C. 1981. Enzymatic synthesis of biotin-labelled polynucleotides : Novel nucleic acid affinity probes. *Proceedings of the National Academy of Sciences USA* **78**: 6633-6637.

Laux,G., Freese,U.K. & Bornkamm,G.W. 1985. Structure and evaluation of two related transcription units of Epstein-Barr virus carrying small tandem repeats. *Journal of Virology* **56**: 987-995.

Lawrence,J.B. & Singer,R.H. 1985. Quantitative analysis of *in situ* hybridisation methods for the detection of actin gene expression. *Nucleic Acids Research* **13**: 1777-1799.

Leary,J.J., Brigati,D.J. & Ward,D.C. 1983. Rapid and sensitive colorimetric method for visualising biotin-labelled DNA probes hybridised to DNA or RNA immobilised on nitrocellulose : Bioblots. *Proceedings of the National Academy of Sciences (USA)* **80**: 4045-4049.

Lederman,L., Kawasaki,E.S. & Szabo,P. 1981. The rate of nucleic acid annealing to cytological preparations is increased in the presence of dextran sulphate. *Analytical Biochemistry* **117**: 158-163.

Lee,W., Hasslinger,A., Karin,M. & Tjian,R. 1987a. Activation of transcription by two factors that bind promoter and enhancer sequences of human metallothionein gene and SV40. *Nature (London)* **325**: 349-359.

- Lee,W., Mitchell,P. & Tjian,R. 1987b. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* **41**: 349-359.
- Lewis,F.A., Griffiths,S., Dunnicliff,R., Wells,M., Dudding,N. & Bird,C.C. 1987. Sensitive *in situ* hybridisation technique using biotin-streptavidin-polyalkaline phosphatase complex. *Journal of Clinical Pathology* **40**: 163-166.
- Lewis,M.E., Sherman,T.G., Burke,S., Akil,H., Davis,L.G., Arentzen,R. & Watson,S.J. 1986. Detection of proopiomelanocortin mRNA by *in situ* hybridisation with an oligonucleotide probe. *Proceedings of the National Academy of Sciences (USA)* **83**: 5419-5423.
- Lieberman,P.M., O'Hare,P. & Hayward,G.S. 1986. Promiscuous *trans*-activation of gene expression by a Epstein-Barr virus-encoded early nuclear protein. *Journal of Virology* **60**: 140-148.
- Loning,T., Milde,K. & Foss,H.D. 1986. *In situ* hybridisation for the detection of cytomegalovirus (CMV) infection. Application of biotinylated CMV-DNA probes on paraffin-embedded specimens. *Virchow's Archiv* **409**: 777-790.
- Love,A., Rydbeck,R., Utter,G., Orvell,C., Kristensson,K. & Norrby,E. 1986. Monoclonal antibodies against the fusion protein are protective in necrotising mumps meningoencephalitis. *Journal of Virology* **58**: 220-222.
- Mackenzie,C.D., Taylor,P.M. & Askonas,B.A. 1989. Rapid recovery of lung histology correlates with clearance of influenza virus by specific CD8<sup>+</sup> cytotoxic T cells. *Immunology* **67**: 375-381.
- McAllister,H.A. & Rock,D.L. 1985. Comparative usefulness of tissue fixatives for *in situ* viral nucleic acid hybridisation. *Journal of Histochemistry & Cytochemistry* **33**: 1026-1032.
- McCabe,J.T., Morrell,J.I., Ivell,R., Schmale,H., Richter,D. & Pfaff,D.W. 1986. *In situ* hybridisation technique to localise rRNA and mRNA in mammalian neurons. *Journal of Histochemistry and Cytochemistry* **34**: 45-50.
- McChesney,M.B. & Oldstone,M.B.A. 1987. Viruses perturb lymphocyte functions : selected principles characterising virus-induced immunosuppression. *Annual Review of Immunology* **5**: 279-304.

McChesney,M.B., Fujinami,R.S., Lampert,P.W. & Oldstone,M.B.A. 1986. Viruses disrupt functions of human lymphocytes. II. Measles virus suppresses antibody production by acting on B lymphocytes. *Journal of Experimental Medicine* **163**: 1331-1336.

McConaughy,B.L., Laird,C.D. & McCarthy,B.J. 1969. Nucleic acid reassociation in formamide. *Biochemistry* **8**: 3289-3295.

McDougall,J.K., Myerson,D. & Beckmann,A.M. 1986. Detection of viral DNA and RNA by *in situ* hybridisation. *Journal of Histochemistry and Cytochemistry* **34**: 33-38.

McSheehey,P. & Chambers,T. 1986. Osteoblast-like cells in the presence of parathyroid hormone release soluble factors that stimulate osteoclastic bone resorption. *Endocrinology* **119**: 1654-1659.

Mead,D.A., Szczesna-Skorupa,E. & Kemper,B. 1986. Single-stranded DNA 'blue' T7 promoter plasmids : a versatile tandem promoter system for cloning and protein engineering. *Protein Engineering* **1**: 67-74.

Meinkoth,J. & Wahl,G. 1984. Hybridisation of nucleic acids immobilised on solid supports. *Analytical Biochemistry* **138**: 267-284.

Melendez,L.V., Daniel,M.D., Hunt,R.D. & Garcia,F.G. 1968. An apparently new herpesvirus from primary kidney cultures of the squirrel monkey (*Saimiri sciureus*). *Laboratory Animal Care* **18**: 374-381.

Melendez,L.V., Hunt,R.D., Daniel,M.D., Garcia,F.G. & Fraser,C.E.O. 1969. Herpesvirus saimiri. II. Experimentally induced malignant lymphoma in primates. *Laboratory Animal Care* **19**: 378-386.

Melton,D., Krieg,P., Rebagliati,M., Maniatis,T., Zinn,K. & Green,M.R. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridisation probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Research* **12**: 7035-7056.

Messing,J. 1983. New M13 vectors for cloning. *Methods in Enzymology* **101**: 20-78.

Mills.B.G. & Singer,F.R. 1976. Nuclear inclusions in Paget's disease of bone. *Science* **194**: 201-202.

- Mills,B.G. & Singer,F.R. 1987. Critical evaluation of viral antigen data in Paget's disease of bone. *Orthopaedic & Related Research* **217**: 16-25.
- Mills,B.G., Singer,F.G., Werner,L.P. & Holst,P.A. 1981. Immunohistological demonstration of respiratory syncytial virus antigens in Paget's disease of bone. *Proceedings of the National Academy of Sciences (USA)* **78**: 1209-1213.
- Mills,B.G., Singer,F.R., Werner,L.P., Suffen,S.C., Stabile,E. & Holst,P. 1984. Evidence for both respiratory syncytial virus and measles virus antigens in the osteoclasts of patients with Paget's disease of bone. *Clinical Orthopaedics & Related Research* **183**: 303-311.
- Mills,B.G., Stabile,E., Holst,P.A. & Graham,C. 1982. Antigens to two different viruses in Paget's disease of bone. *Journal of Dental Research* **61**: 341-351.
- Minagawa,T., Sakuma,T., Kuwajima,S., Yamamoto,T.K. & Iida,H. 1976. Characterisation of measles virus in establishment of persistent infections in human lymphoid cell line. *Journal of General Virology* **33**: 361-379.
- Moar,M.H. & Klein,G. 1978. Detection of Epstein-Barr virus (EBV) DNA sequences using *in situ* hybridisation. *Biochimica et Biophysica Acta* **519**: 49-64.
- Modrow,S. & Wolf,H. 1983. Herpesvirus saimiri-induced proteins in lytically infected cells. I. Time-ordered synthesis. *Journal of General Virology* **64**: 37-46.
- Morein,B. 1988. The iscom antigen-presenting system. *Nature (London)* **332**: 287-288.
- Morein,B., Sundquist,B., Hoglund,S., Dalsgaard,K. & Osterhaus,A. 1984. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature* **308**: 457-460.
- Morgan,D.G. 1977. Observations on the antigenic relationships between Epstein-Barr virus and herpesvirus saimiri. *Journal of General Virology* **36**: 281-287.
- Moss,B., Fuerst,T.R., Flexner,C. & Hugin,A. 1988. Roles of vaccinia virus in the development of new vaccines. *Vaccine* **6**: 161-163.



Mullink,H., Walboomers,J.M.M., Raap,A.K. & Meyer,C.J.L.M. 1989. Two colour DNA *in situ* hybridisation for the detection of two viral genomes using non-radioactive probes. *Histochemistry* 91: 195-198.

Murthy,S.C.S., Trimble,J.J. & Desrosiers,R.C. 1989. Deletion mutants of herpesvirus saimiri define an open reading frame necessary for transformation. *Journal of Virology* 63: 3307-3314.

Neel,B.G., Jhanwar,S.C., Chaganti,R.S.K. & Hayward,W.S. 1982. Two human c-onc genes are located on the long arm of chromosome 8. *Proceedings of the National Academy of Sciences (USA)* 79: 7842-7846.

Nicholas,J., Gompels,U.A., Craxton,M.A. & Honess,R.W. 1988. Conservation of sequence and function between the products of the 52-kilodalton immediate-early gene of HVS and the BMLF-1-encoded transcriptional effector (EB2) of EBV. *Journal of Virology* 62: 3250-3257.

Nicholas,J., Smith,E.P., Coles,L. & Honess,R.W. 1990. Gene expression in cells infected with gammaherpesvirus saimiri : properties of transcripts from two immediate-early genes. *Journal of General Virology*. In press.

Norrby,E. 1978. Viral antibodies in multiple sclerosis. *Progress in Medical Virology* 24: 1-39.

Norrby,E., Kristensson,K., Brzosko,W.J. & Kapsenberg,J.G. 1985. Measles virus matrix protein detected by immune fluorescence with monoclonal antibodies in the brain of patients with subacute sclerosing panencephalitis. *Journal of Virology* 56: 337-340.

O'Driscoll,J.B. & Anderson,D.C. 1985. Past pets and Paget's disease. *Lancet* II: 907-913.

Ohuchi,M., Ohuchi,R., Mifune,K., Ishihara,T. & Ogawa,T. 1987. Characterisation of the measles virus isolated from the brain of a patient with immunosuppressive measles encephalitis. *Journal of Infectious Diseases* 156: 436-441.

Oldstone,M.B.A. 1989. Viral persistence. *Cell* 56: 517-520.

Oldstone, M.B.A. & Tishon, A. 1978. Immunologic injury in measles virus infection. IV. Antigenic modulation and abrogation of lymphocyte lysis of virus-infected cells. *Clinical Immunology and Immunopathology* 9: 55-62.

Osdoby, P., Krukowski, M., Oursler, M.J. & Salino-Hugg, T. 1987. The origin, development and regulation of osteoclasts. *Bioessays* 7: 30-34.

Paget, J. 1877. On a form of chronic inflammation of the bones (Osteitis deformans). *Med. Chir. Trans. LX*: 37-63.

Patzer, E.J., Wagner, R.R. & Dubone, E.J. 1979. Viral membranes : model systems for studying biological membranes. *Critical Reviews in Biochemistry* 6: 165-217.

Pearce, A.G.E. 1985. *Histochemistry Theoretical and Applied. Analytical Technology. Volume II.* Churchill Livingstone.

Perlman, S., Jacobson, G. & Moore, S. 1988. Regional localisation of virus in the central nervous system of mice persistently infected with murine coronavirus JHM. *Virology* 166: 328-338.

Pettay, O., Donner, M., Halonen, H., Palosuo, T. & Salmi, A. 1971. Subacute sclerosing panencephalitis : Preceding intellectual deterioration and deviant measles serology. *Journal of Infectious Diseases* 124: 439-444.

Pezzella, M., Rossi, P., Lombardi, V., Gemelli, V., Mariani Costantini, R., Mirolo, M., Fundaro, C., Moschese, V. & Wigzell, H. 1989. HIV viral sequences in seronegative people at risk detected by *in situ* hybridisation and polymerase chain reaction. *British Medical Journal* 298: 713-716.

Preble, O.T. & Youngner, J.S. 1973a. Temperature-sensitive defect of mutants isolated from L cells persistently infected with Newcastle disease virus. *Journal of Virology* 12: 472-480.

Preble, O.T. & Youngner, J.S. 1973b. Selection of temperature-sensitive mutants during persistent infections : Role in maintenance of persistent Newcastle disease virus infections of L cells. *Journal of Virology* 12: 481-491.

Rammohan, K.W., McFarland, H.F. & McFarlin, D.E. 1982. Subacute sclerosing panencephalitis after passive immunisation and natural measles infection. Role of antibody in persistence of measles virus. *Neurology* 32: 390-394.

Randall,R.E. 1989. Solid matrix-antibody-antigen (SMAA) complexes for constructing multivalent subunit vaccines. *Immunology Today* 10: 336-339.

Randall,R.E., Honess,R.W. & O'Hare,P. 1983. Proteins specified by herpesvirus saimiri : identification and properties of virus-specific polypeptides in productively infected cells. *Journal of General Virology* 64: 19-35.

Randall,R.E., Newman,C. & Honess,R.W. 1984a. Isolation and characterisation of monoclonal antibodies to structural and non-structural herpesvirus saimiri proteins. *Journal of Virology* 52: 872-883.

Randall,R.E., Newman,C. & Honess,R.W. 1984b. A single major immediate-early virus gene product is synthesised in cells productively infected with herpesvirus saimiri. *Journal of General Virology* 65: 1215-1219.

Randall,R.E., Newman,C. & Honess,R.W. 1985. Asynchronous expression of the immediate-early protein of herpesvirus saimiri in populations of productively infected cells. *Journal of General Virology* 66: 2199-2213.

Randall,R.E. & Young,D.F. 1988. Humoral and cytotoxic T cell immune response to internal and external structural proteins of simian virus 5 induced by immunisation with solid matrix-antibody-antigen complexes. *Journal of General Virology* 69: 2505-2516.

Randall,R.E. & Young,D.F. 1989. Immunisation against multiple viruses by using solid matrix-antibody-antigen complexes. *Journal of Virology* 63: 1808-1810.

Randall,R.E., Young,D.F. & Southern,J.A. 1988. Immunisation with solid matrix-antibody-antigen complexes containing surface or internal virus structural proteins protects mice from infection with the paramyxovirus, simian virus 5. *Journal of General Virology* 69: 2517-2526.

Rangan,S.R.S., Martin,L.N., Enright,F.M. & Allen,W.P. 1976. Herpesvirus saimiri-induced malignant lymphoma in rabbits. *Journal of the National Cancer Institute* 57: 151-156.

Rebel,A., Basle,M.F., Pouplard,A., Kongourndjian,S., Filman,R. & Lepakezour,A. 1980a. Viral antigens in osteoclasts from Paget's disease of bone. *Lancet* I: 334-346.

Rebel,A., Basle,M.F., Pouplard,A., Malkani,K., Filmon,R. & Lepatezour,A. 1980b. Bone tissue in Paget's disease of bone. Ultrastructure and immunocytoology. *Arthritis & Rheumatism* **23**: 1104-1114.

Rebel,A., Malkani,K., Basle,M. & Bregonc,C. 1977. Is Paget's disease of bone a viral infection? *Calcified Tissue Research* **22**: 283-286.

Reinherz,E., Kamp,P.F., Goldstein,G. & Schlossman,S.F. 1979. Separation of functional subsets of human T cells using monoclonal antibodies. *Proceedings of the National Academy of Sciences (USA)* **76**: 4061-4064.

Ricca,G.A., Taylor,J.M. & Kalinyak,J.E. 1982. Simple rapid method for the synthesis of radioactively labelled cDNA hybridisation probes utilising bacteriophage M13mp7. *Proceedings of the National Academy of Sciences (USA)* **79**: 724-728.

Rigby,P.W.J., Dieckmann,M., Rhodes,C. & Berg,P. 1977. Labelling DNA to high specific activity *in vitro* by nick translation with DNA polymerase I. *Journal of Molecular Biology* **113**: 237-251.

Riley,L.K., Marshall,M.E. & Coleman,M.S. 1986. A method for biotinylating oligonucleotide probes for use in molecular hybridisations. *DNA* **3**: 333-337.

Rima,B.K., Baczko,K., Clark,D.K., Curran,M.D., Martin,S.J., Billeter,M. & ter Meulen,V. 1986. Characterisation of clones for the sixth (L) gene and a transcriptional map for morbilliviruses. *Journal of General Virology* **67**: 1971-1978.

Rima,B.K., Davidson,W.B. & Martin,S.J. 1977. The role of defective interfering particles in persistently infection of Vero cells by measles virus. *Journal of General Virology* **35**: 89-97.

Rima,B.K. & Martin,S.J. 1976. Persistent infection of tissue culture cells by RNA viruses. *Medical Microbiology & Immunology* **162**: 89-118.

Risk,W.S., Haddad,F.S. & Chemali,R. 1978. Substantial spontaneous long-term improvement in sub-acute sclerosing panencephalitis. *Archives of Neurology* **35**: 494-502.

Robertson,D.A.F., Zhang,S.L., Fawcner,K., Alvey,C., Hu,X-Y. & Wright,R. 1989. Measles vaccination may have prevented 'autoimmune' chronic active hepatitis in young people. *Gastroenterology*. In press.

Robertson,D.A.F., Zhang,S.L., Guy,E.C. & Wright,R. 1987. Persistent measles virus genome in autoimmune chronic active hepatitis. *Lancet* II: 9-11.

Rock,D.L., Nesburn,A.B., Ghiasi,H., Ong,J., Lewis,T.L., Lokensgard,J.R. & Wechsler,S.L. 1987. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. *Journal of Virology* 61: 3820-3826.

Rooney,C., Taylor,N., Countryman,J., Jenson,H., Kolman,J. & Miller,G. 1988. Genome rearrangements activate the Epstein-Barr virus gene whose product disrupts latency. *Proceedings of the National Academy of Sciences (USA)* 85: 9801-9805.

Roux,L. & Holland,J.J. 1979. Role of defective interfering particles of Sendai virus in persistent infections. *Virology* 93: 91-103.

Rozenblatt,S., Eizengerg,O., Ben-Levy,R., Lavie,V. & Bellini,W.J. 1985. Sequence homology within the morbilliviruses. *Journal of Virology* 53: 684-690.

Russell,W.C. 1983. Paramyxovirus and morbillivirus infections and their relationship to neurological disease. *Progress in Brain Research* 59: 113-132.

Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S., Higuchi,R., Horn,G.T., Mullis,K.B. & Erlich,H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.

Saiki,R.K., Scharf,S., Faloona,F., Mullis,K.B., Horn,G.T., Erlich,H.A. & Arheim,N. 1985. Enzymatic synthesis of  $\beta$ -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230: 1350-1354.

Salmi,A.A., Norrby,E. & Panelius,M. 1972. Identification of different measles virus-specific antibodies in the serum and cerebrospinal fluid from patients with sub-acute sclerosing panencephalitis and multiple sclerosis. *Infection & Immunity* 6: 248-254.



Scheid,A & Choppin,P.W. 1974. Identification of the biological activities of paramyxovirus glycoproteins. Activation of cell fusion hemolysis and infectivity by proteolytic cleavage of an inactive precursor protein of Sendai virus. *Virology* 57: 475-490.

Scheid,A., Caligniri,L.A., Compans,R.W. & Choppin,P.W. 1972. Isolation of paramyxovirus glycoproteins. Association of both haemagglutinin and neuraminidase activities with the largest SV5 glycoprotein. *Virology* 50: 640-652.

Schulman,J.L., Khakpour,M. & Kilbourne,E.D. 1968. Protective effects of specific immunity to viral neuraminidase on influenza virus infection of mice. *Journal of Virology* 2: 778-786.

Shapsak,P., Tourtellotte,W.W., Nakamura,S., Graves,M.C., Darvish,M., Hoffman,D., Walsh,M.J., Fareed,G.C., Schmid,P., Heinzmann,C., Sidhu,K., Bedows,E., Rozenblatt,S., Berry,K. & Hawkins,S.1985. Subacute sclerosing panencephalitis : measles virus matrix protein nucleic acid sequences detected by *in situ* hybridisation. *Neurology* 35: 1605-1609.

Shioda,T., Iwasaki,K. & Shibuta,H. 1986. Determination of the complete nucleotide sequence of the Sendai virus genome RNA and the predicted amino acid sequences of the F, HN and L proteins. *Nucleic Acids Research* 14: 1545-1563.

Singer,F.R. 1980. Paget's disease of bone : A slow virus infection ? *Calcified Tissue International* 31: 185-187.

Singer,R.H., Langevin,G.L. & Lawrence,J.B. 1986. Electron microscopic visualisation of single intracellular actin messenger RNA molecules by *in situ* hybridisation. *Journal of Cell Biology* 103: 315.

Singer,R.H., Lawrence,J.B., Langevin,G.L., Rashtchian,R.N., Villnave,C.A., Cremer,T. Tesin,D., Mannelidish,L. & Ward,D.C. 1987. Double-labelling *in situ* hybridisation using non-isotopic and isotopic detection. *Acta Histochemistry & Cytochemistry* 20: 589-598.

Singer,R.H., Lawrence,J.B., Silva,F., Langevin,G.L., Pomeroy,M. & Billings-Gagliardi,S. 1989. Strategies for ultrstructural visualisation of biotinylated probes hybridised to messenger RNA *in situ*. In "Current Topics in Microbiology & Immunology." Volume 143. Eds. Haase,A.T. & Oldstone,M.B.A. Springer-Verlag : Berlin. pp55-69.

Singer, F.R. & Mills, B.G. 1983. Evidence for viral etiology of Paget's disease of bone. *Clinical Orthopaedics & Related Research* 178: 245-251.

Singer, R.H. & Ward, D.C. 1982. Actin gene expression visualised in chicken muscle culture by using *in situ* hybridisation with a biotinated nucleotide analog. *Proceedings of the National Academy of Sciences (USA)* 79: 7331-7335.

Spriggs, M.K., Olmsted, R.A., Venkatesan, S., Coligan, J.E. & Collins, P.L. 1986. Fusion protein of human parainfluenza virus type 3 : nucleotide sequence of the gene, direct identification of the cleavage-activation site, and comparison with other paramyxoviruses. *Virology* 152: 241-251.

Staminger, T.R., Honess, R.W., Young, D.F., Bodemer, W. Blair, E.D. & Fleckenstein, B. 1987. Organisation of terminal reiterations in the virion DNA of herpesvirus saimiri. *Journal of General Virology* 68: 1049-1066.

Steinhauer, D.A. & Holland, J.J. 1987. Rapid evolution of RNA viruses. *Annual Review of Microbiology* 41: 409-433.

Stevens, J.G. & Cook, M.L. 1971. Latent herpes simplex virus in sensory ganglia. *Science* 173: 843-845.

Stevens, J.G., Haarr, L., Portner, D.D., Cook, M.L. & Wagner, E.K. 1988. The herpes simplex virus latency-associated transcript is prominent in trigeminal ganglia from seropositive humans. *Journal of Infectious Diseases* 158: 117-123.

Stevens, J.G., Wagner, E.K., Devi-Roa, G.B., Cook, M.L. & Feldman, L.T. 1987. RNA complementary to a herpesvirus  $\alpha$  gene is prominent in latently infected neurons. *Science* 235: 1056-1059.

Stott, E.J. & Taylor, G. 1984. Respiratory syncytial virus. A review. *Archives of Virology* 84: 1-52.

Stowring, L., Haase, A.T., Petursson, G., Georgsson, G., Palsson, P., Lutley, R., Roos, R. & Szuchet, S. 1985. Detection of visna virus antigens and RNA in glial cells in foci of demyelination. *Virology* 141: 311-318.

Stroop,W.G., Rock,D.L. & Fraser,N.W. 1984. Localisation of herpes simplex virus in the trigeminal and olfactory systems of the mouse central nervous system during acute and latent infections by *in situ* hybridisation. *Laboratory Investigations* 51: 27-38.

Studenki,A.B. & Wallace,R.B. 1984. Allele-specific hybridisation using oligonucleotide probes of very high specific activity : Discrimination of the human  $\beta^A$  and  $\beta^S$ -globin genes. *DNA* 3: 7-15.

Szabo,P., Elder,R., Steffensen,D.M. & Uhlenbeck,O.C. 1977. Quantitative *in situ* hybridisation of ribosomal RNA species to polytene chromosomes of *Drosophila melanogaster*. *Journal of Molecular Biology* 115: 539-563.

Taylor,P.M. & Askonas,B.A. 1986. Influenza nucleoprotein-specific cytotoxic T cell clones are protective *in vivo*. *Immunology* 58: 417-420.

Taylor,G., Stott,E.J., Bew,M., Fernie,B.F., Cote,P.J., Collins,A.P. Hughes,M. & Jebbett,J. 1984. Monoclonal antibodies protect against respiratory syncytial virus infection in mice. *Immunology* 52: 137-142.

Taylor,G., Stott,E.J. & Hayle,A.J. 1985. Cytotoxic lymphocytes in the lungs of mice infected with respiratory syncytial virus. *Journal of General Virology* 66: 2533-2538.

Tellez-Nagel,J. & Harter,D.H. 1966. Subacute sclerosing leucoencephalitis : Ultrastructure of intranuclear inclusions. *Science* 154: 899-900.

Tenser,R.B., Dawson,M., Ressel,S.J. & Dunston,M.E. 1982. Detection of herpes simplex virus mRNA in latently infected trigeminal ganglion neurons by *in situ* hybridisation. *Annals of Neurology* 11: 285-291.

ter Meulen,V. & Hall,W.W. 1978. Slow virus infections of the nervous system : virological, immunological and pathogenic considerations. *Journal of General Virology* 41: 1-25.

ter Meulen,V., Muller,D & Joppich,C. 1967. Fluorescence microscopy studies of brain tissue from a case of sub-acute sclerosing panencephalitis. *German Medical Methods* 12: 438.

ter Meulen,V. & Stephenson,J.R. 1981. The possible role of virus infections in multiple sclerosis and other related diseases. In "Multiple sclerosis : Pathology, Diagnosis and Management". Eds. Haupike,J.F., Adams,C.W.M. & Tourtellotte,W.W. Williams & Williams: Baltimore,Maryland. pp379-399.

Thomas,C.A. & Dancis,B.M. 1973. Ring stability. *Journal of Molecular Biology* 73: 43-55.

Trent,J.M., Olson,S. & Lawn,R.M. 1982. Chromosomal localisation of human leucocyte, fibroblast and immune interferon genes by means of *in situ* hybridisation. *Proceedings of the National Academy of Sciences (USA)* 79: 7809-7813.

Triger,D.R., Kurtze,J.B., MacCallum,P.O. & Wright,R. 1972. Raised antibody titres to measles and rubella viruses in chronic active liver hepatitis. *Lancet* i: 665-667.

Truant,A.L. & Hallum,J.V. 1977. A persistent infection of baby hamster kidney-21 cells with mumps virus and the role of the temperature-sensitive variants. *Journal of Medical Virology* 1: 49-67.

Tyrrell,D.L.J., Rafler,D.J., Orvell,C. & Norrby,E. 1980. Isolation and immunological characterisation of the nucleocapsid and membrane proteins of measles virus. *Journal of General Virology* 51: 307-315.

Uhl,G.R., Zingg,H.H. & Habbener,J.F. 1985. Vasopressin mRNA *in situ* hybridisation : localisation and regulation studied with oligonucleotide cDNA probes in normal and Brattleboro rat hypothalamus. *Proceedings of the National Academy of Sciences (USA)* 82: 5555-5559.

Unger,E.R. & Brigati,D.J. 1989. Colorimetric *in situ* hybridisation in clinical virology. In "Current Topics in Microbiology." Volume 143. Eds. Haase,A.T. & Oldstone,M.B.A. Springer-Verlag : Berlin. pp21-31.

Unger,E.R., Budgeon,L.R., Myerson,D. & Brigati,D.J. 1986. Viral diagnosis by *in situ* hybridisation : description of a rapid simplified colorimetric method. *American Journal of Surgical Pathology* 10: 1-8.

Vandvik,B. 1973. Immunopathological aspects in the pathogenesis of sub-acute sclerosing panencephalitis, with special reference to the significance of the immune response in the central nervous system. *Annals of Clinical Research* 5: 308-315.

Vandvik,B., Norrby,E., Nordal,N.J. & Degre,M. 1976. Oligoclonal measles virus-specific IgG antibodies isolated from cerebro-spinal fluids, brain extracts and sera from patients with sub-acute sclerosing panencephalitis and multiple sclerosis. *Scandinavian Journal of Immunology* 5: 980-992.

Virelizier,J.L., Oxford,J.S. & Schild,G.C. 1976. The role of humoral antibody in host defence against influenza A infection in mice. *Postgraduate Medical Journal* 52: 332-337.

Wahl,G.M., Stern,M. & Stark,G.R. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzylmethyl-paper and rapid hybridisation by using dextran sulphate. *Proceedings of the National Academy of Sciences (USA)* 76: 3683-3687.

Walker,D. 1975. Bone resorption restored in osteopetrotic mice by transplants of normal bone marrow and spleen cells. *Science* 190: 784-785.

Wallace,R.B. Scaffer,J., Murphy,R.F., Bonner,J., Hirose,T. & Itakura,K. 1979. Hybridisation of synthetic oligodeoxynucleotides to  $\phi$ X174 DNA : the effect of single base pair mismatch. *Nucleic Acids Research* 6: 3543-3557.

Wallace,R.B., Johnson,M.J.m Hirose,T., Miyake,T., Kawashima,E.H. & Itakura,K. 1981. The use of synthetic oligonucleotides as hybridisation probes. II. Hybridisation of oligonucleotides of mixed sequence to rabbit  $\beta$ -globin DNA. *Nucleic Acids Research* 9: 879-894.

Webster,H de F., Lamperth,L., Favilla,J.T., Lemke,G., Tesin,D. & Mannelidis,L. 1987. Use of a biotinylated probe and *in situ* hybridisation for light and electron microscopic localisation of Po mRNA in myelin-forming Schwann cells. *Histochemistry* 86: 441-444.

Wechsler,S.L. & Fields,B.N. 1978. Differences between intracellular polypeptides of measles and sub-acute sclerosing panencephalitis virus. *Nature (London)* 272: 458-460.

Wetmur,J.G. & Davidson,N. 1968. Kinetics of renaturation of DNA. *Journal of Molecular Biology* 31: 349-370.



- Wetmur, J.G., Ruyechan, W.T. & Douthart, R.J. 1981. Denaturation and renaturation of *Penicillium chrysogenum* mycophage double-stranded ribonucleic acid in tetraalkylammonium salt solutions. *Biochemistry* 20: 2999-3002.
- Wilson, T. & Lenard, J. 1981. Interaction of wild-type and mutant protein vesicular stomatitis virus with nucleocapsids *in vitro*. *Biochemistry* 20: 1349-1354.
- Wolfe, L.G., Falk, L.A. & Deinhardt, F. 1971. Oncogenicity of herpesvirus saimiri in marmoset monkeys. *Journal of the National Cancer Institute* 47: 1145-1162.
- Wolinsky, J.S., Waxham, M.N. & Server, A.C. 1985. Protective effects of glycoprotein-specific monoclonal antibodies on the course of experimental mumps virus meningoencephalitis. *Journal of Virology* 53: 727-734.
- Wong, K-M. & Levine, A.J. 1986. Identification and mapping of Epstein-Barr virus early antigens and demonstration of a viral gene activator that functions in *trans*. *Journal of Virology* 60: 149-156.
- Wraith, D.C., Vessey, A.E. & Askonas, B.A. 1987. Purified influenza virus nucleoprotein protects mice from lethal infection. *Journal of General Virology* 68: 433-440.
- Wright, J., Falk, L.A., Collins, D. & Denhardt, F. 1976. Mononuclear cell fraction carrying herpesvirus saimiri in persistently infected squirrel monkeys. *Journal of the National Cancer Institute* 57: 959-962.
- Young, D.F., Randall, R.E., Hoyle, J.A. & Souberbielle, B.E. 1990. Clearance of a persistent paramyxovirus infection is mediated by cellular immune responses but not by serum neutralising antibody. *Journal of Virology*. In press.
- Youngner, J.S. & Preble, O.T. 1980. Virus persistence : Evolution of viral populations. In "Comprehensive Virology". Volume 16. Eds. Fraenkel-Conrat, H. & Wagner, R.R. Plenum Press : New York. pp73-135.
- Zinn, K., DiMaio, D. & Maniatis, T. 1983. Identification of two distinct regulatory regions adjacent to the human  $\beta$ -interferon gene. *Cell* 34: 865-879.
- Zurbriggen, A. & Fujinami, R.S. 1988. Theiler's virus infection in nude mice : Viral RNA in vascular endothelial cells. *Journal of Virology* 62: 3589-3596.

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Signed Jane A. Hoyle Date 21/9/90.

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 on 1<sup>st</sup> OCTOBER 1986 and as a candidate for the degree of Ph.D on 1<sup>st</sup> OCTOBER 1987.

Signed Jane A. Hoyle Date 21/9/90.

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